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Determining the Presence of 5-Fluorouracil in Hamster Saliva by HPLC

Determinação, por CLAE, da Presença do 5-Fluorouracil em Saliva de Hamsters

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Abstract

Introduction: Various methods of analysis for the assay of chemotherapeutic agent 5-Fluorouracil (5-FU) in human and animal biological fluids have previously been reported. However, there is no standardization for detecting 5-FU in the hamsters' saliva that received the chemotherapeutic agent. Objective: Considering that the administration of 5-FU in some way changes the morphology and function of the salivary glands, and that the presence of the chemotherapeutic agents in the oral mucosa may lead to some oral complications, the aim of this study was to determine the presence of 5-FU in the hamsters' saliva that received the chemotherapeutic agent, by means of the High Performance Liquid Chromatography technique (HPCL) since this animal model is used in studies of 5-FU induced oral mucositis and glandular hypofunction. Methods: Twelve animals were divided into 4 groups: CP and CPI, in which the animals received pilocarpine (CP) or pilocarpine + isoproterenol (CPI) and the chemotherapy vehicle intraperitoneally; and Groups QP and QPI, in which the animals received the same secretagogues listed above, and the chemotherapeutic agent 5-FU, respectively. After the secretagogue administration, saliva was collected from all the animals for a period of 60 mins. Subsequently, the saliva was frozen at -80 ˚C for later determination of the chemotherapeutic agent by HPLC. After the the chromatograms analysis, and based on the results obtained, it was possible to identify the presence of 5-FU in the saliva samples from hamsters that received the chemotherapeutic agent intraperitonally, by the HPLC technique.

Keywords: Antimetabolites. Chromatography, High Pressure Liquid. Saliva

Resumo

Vários métodos de análise para o ensaio do quimioterápico 5-Fluorouracil (5-FU) em fluidos biológicos de humanos e animais, foram previamente relatados. No entanto, não há uma padronização para detecção de 5-FU na saliva de hamsters que receberam o quimioterápico. Considerando que a administração do 5-FU altera de alguma maneira a morfologia e função das glândulas salivares, e que a presença do quimioterápico na mucosa oral pode levar a algumas complicações orais, este trabalho teve como objetivo de determinar a presença de 5-FU na saliva de hamsters que receberam o quimioterápico pela técnica de Cromatografia Líquida de Alta Eficiência (CLAE), uma vez que este modelo animal é usado nos estudos com mucosite oral e hipofunção glandular, induzidas por 5-FU. Doze animais foram divididos em 4 grupos: CP e CPI, onde os animais receberam intraperitonealmente pilocarpina (CP) ou pilocarpina + isoproterenol (CPI) e o veículo do quimioterápico, e os grupos QP e QPI, onde os animais receberam, respectivamente, os mesmos secretagogos listados acima e o quimioterápico 5-FU. Após a administração do secretagogo, foi coletada a saliva de todos os animais, por um período de 60 min. Em seguida, a saliva foi congelada a -80 ˚C para posterior determinação do quimioterápico por CLAE. Após análise dos cromatogramas, e com base nos resultados obtidos, foi possível identificar a presença do 5-FU nas amostras de saliva de hamsters que receberam o quimioterápico via intraperitoneal pela técnica da CLAE.

Palavras-chave: *Antimetabólitos. Cromatografia Líquida de Alta Pressão. Saliva.*

1 Introduction

Saliva testing to detect various drugs, such as chemotherapeutic agents, has been the objective of study by various authors in an endeavor to correlate the results of salivary analyses with those of analyses performed in plasma[1-6]. The saliva collection method has the advantage of being a noninvasive technique, without exposing patients to discomfort, skin irritation and risk of infection, when compared with plasma collection. Moreover, some authors have correlated the presence of some chemotherapeutic agents in saliva with oral complications resulting from their direct action on the oral mucosa[1,7,8].

However, it is not known yet whether direct contact of chemotherapeutic agents with the oral mucosa could be related to the appearance of oral mucositis, and what effect this drug has on the salivary glands. Although many studies demonstrated that drugs are excreted into the saliva in addition to the blood and urine, a few of them have examined the effect of drugs in saliva on the oral mucosa[9].

Drug toxicity seems to be directly linked to reduced cell turnover in the epithelium basal layer, resulting in desquamation, ulceration, inflammation and atrophy[8,10,11]. Antineoplastic drugs may have a direct effect on the oral mucosa by the secretion of chemotherapeutic substances in

the saliva, or indirectly by the suppression of the immune cells production in the bone marrow and the exposure of the oral mucosa to drugs seems to contribute to the development of pathologies such as mucositis, xerostomia and gingival bleeding[12].

Innumerable cytotoxic agents have been related to the development of damage to the oral and gastrointestinal mucosa, among them, antimetabolite agents (mercaptopurine; cytarabine; 5-Fluorouracil (5-FU) in high doses); plant-derived substrates (etoposide); antitumor agents (doxorubicin), and alkylating agents (melphalan and busulfan). The agent 5-FU ($C_4H_3FN_2O_2$), a polar molecule, was introduced into the therapeutic clinic for the treatment of tumors in 1957. Currently, it is still used in the treatment of gastric, and colorectal tumors; tumors of the head and neck; breast; ovaries; prostate; liver; and of the genitourinary tract[13].

The 5-FU, is an antimetabolite analog of Uracil (U) that enters the cell using the same facilitated transport of U with a fluoride atom in position C-5 in the place of hydrogen. There are three different cytotoxic form attributed to 5-FU: incorporation of fluoronucleotides into DNA or RNA, which triggers the apoptosis process; and inhibition of the thymidylate synthetase enzyme (TS). TS is a target enzyme of 5-FU and the increase in its expression is a potential mechanism of resistance to this drug[14].

Various analytical methods to test for 5-FU chemotherapy in biological fluids have previously been reported, including the separation of the structurally related compounds, its metabolites, such as the pyrimidines, and especially uracil, that has a retention time close to that of 5-FU[15]. For this purpose, the authors used High Performance Liquid Chromatography, from which the acronym HPLC is derived. However, standardization of the 5-FU detection technique in hamster saliva by means of HPLC, may - in future studies - help with understanding how some oral complications occur after administration of this chemotherapeutic agent.

Therefore, the aim of this study was to determine the presence of the chemotherapeutic agent 5-FU, by HPLC, in the hamsters' saliva that received the drug intraperitoneally.

2 Material and Methods

The protocol used in this study was approved by the Ethics Committee on the Use of Animals of the University of São Paulo (USP) Dental School (FOUSP), number 004/2013, and is in accordance with the Ethical Principles of Animal Experimentation, adopted by the Brazilian Society of Laboratory Animal Science (SBCAL).

Golden Syrian Hamsters (males) were used, with body mass between 120 to 200 grams. First the animals were weighed and individually separated in cages with access to water and food ad libitum. After this, they were divided into different groups, according to the use of the secretagogue and 5-FU, as follows:

Group CP: Animals received pilocarpine and vehicle for chemotherapy;
Group CPI: Animals received pilocarpine + isoproterenol and vehicle for chemotherapy;
Group QP: Animals received pilocarpine and 5-FU injection;
Group QPI: Animals received pilocarpine + isoproterenol and 5-FU injection;

Before the 5-FU injection process, the animals were anesthetized with an association of Anasedan (Ceva, Paulínia, Brazil) 13.8 mg/kg and Dopalen (Ceva, Paulínia, Brazil) 11.6 mg/kg, and after that , they received an intraperitoneal injections of solutions of Pilocarpine (7.5mg/kg p.c) (Sigma-Aldrich®, St. Louis, MO, USA), and Isoproterenol (5.0 mg/kg p.c.) (Sigma-Aldrich®, St. Louis, MO, USA), dissolved in distilled water, to stimulate salivation. After doing this, 65 mg/kg of 5-FU (Sigma-Aldrich®, St. Louis, MO, USA), diluted in ammonium hydroxide 1M (vehicle), was injected intraperitoneally, according to the protocol previously described[10]. The animals in the control group received the vehicle only.

Saliva samples were collected for a period of 60 minutes. To collect saliva, a funnel was used, with the purpose of directing the salivary flow into the falcon collection tube that was kept on crushed ice. After collection, the samples were stored in a freezer at - 80 ºC until the time of analyses. After the collection time, the animals were sacrificed by severing the spinal cord.

2.1 Saliva sample preparation

Before chromatographic analysis, the saliva samples of Groups CP, CPI, QP and QPI were defrosted and prepared according to the method cited by Joulia et al, in which to each aliquot of 100 µl of saliva, 20 µl of the internal standard solution (Bromouracil, 5 µg/ml) was added and carefully mixed, adding 100µl of acetonitrile, a polar solvent, and then the sample was centrifuged (10 min at 1350 g and 4 ºC). The supernatant was transferred to a test tube and each 100µl, was mixed with 300 µl of ethyl acetate. The mixture was then centrifuged (10 min at 1540 g and 4 ºC). The superior organic phase was transferred by pipette to a glass test tube, for evaporation in Nitrogen until dry, at a Temperature fixed at 40 ºC. The dry residue was resuspended in 200 µl of deionized water, and subsequently the samples were filtered through Millex® filters, 0.45µm (Millipore) before injection into the column[6].

2.2 Analytical Method

The analyses were performed in a High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan) according to the official monograph USP 38 NF 33, 2015[16]. The mentioned method was modified concerning the proportion of the mobile phase and chromatography column dimensions to optimize retention time and availability of materials, respectively. Therefore, a reverse phase column (C18, 150 x 4,6 mm, 3 µm; Ace,

Scotland) was used, and the mobile phase consisted of 100% phosphate buffer solution 0.05 M, with isocratic elution, diluted in deionized water. The pH of the solution was adjusted to 5.7 ± 0.1, with a potassium hydroxide solution (KOH) 5M. The mobile phase flow was adjusted to 1.0 mL/min. Sample injection, with a volume of 20 µl, was performed manually. The diode array detector (DAD) was used for detection at a wavelength of 254 nm.

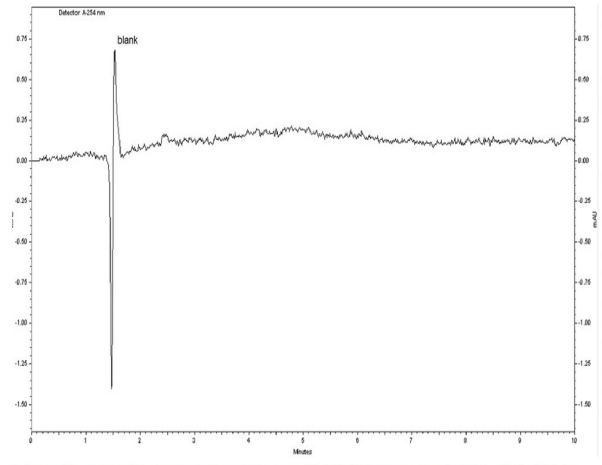
White and standard 5-FU curves were prepared. The white curve used was deionized water For the 5-FU standard curve, 10 mg of the reference substance for 5-FU was weighed (Sigma-Aldrich®, St. Louis, MO, USA) and transferred to a 1L amber volumetric flask (VF), thus obtaining a concentration of 10 µg/mL of the analyte. From this solution, the concentrations of 0.1, µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL and 7.5µg/mL were obtained. In addition, an internal standard 5-BU sample was prepared (Sigma-Aldrich®, St. Louis, MO, USA), at the concentration of 5 µg/mL.

After the samples and the standard solutions of 5-FU and 5-BU were prepared and were sequentially injected into the equipment, according to the description: blank solution; internal standard solution (5µg/ml, 5-BU; a known concentration of the 5-FU solution; blank solution; samples from Groups CP and CPI, animals that did not receive the chemotherapeutic agent; a known concentration of the 5-FU solution; samples from Groups QP and QPI, animals that received 5-FU.

3 Results and Discussion

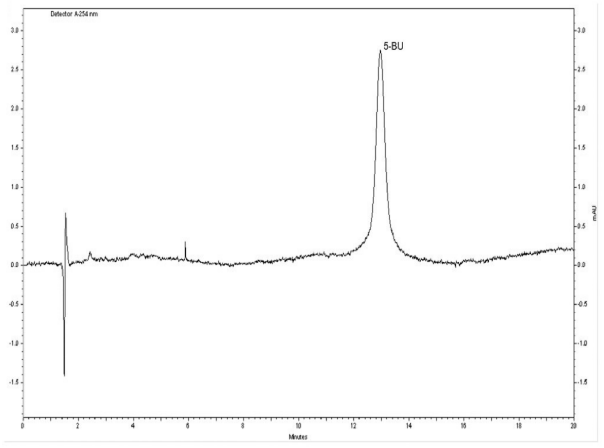
The choromatographic results were subdivided into the white identification; internal standard (5-BU), standard (5-FU) and in the sequence of the different Groups (CP, CPI, QP and QPI). Figure 1 represents the white sample chromatogram, deionized water, in which no peak was observed in the regions of 5-FU and 5-BU. Figure 2 represents the chromatograph of the internal standard solution 5-Bromouracil (5-BU)m and this presented a retention time (rt) of 13 ± 0.5 minutes for all the readouts. Figures 3 and 4 represent the chromatogram of the 5-FU solution, and demonstrated that the rt of 5-FU, even when the concentrations were varied from 10.000 ng/ml to 100 ng/ml, were maintained at 4.6 ± 0.4 minutes for all the readouts. Figures 5 and 6 represent the chromatograms of saliva samples from Groups CP and CPI, animals that did not receive chemotherapy, but received the internal standard during treatment of the samples. After taking the readouts, the peak in rt o 5-BU and absence of rt of 5-FU was observed. Figures 7 and 8 represent the chromatograms of saliva samples from Groups QP and QPI, animals that received chemotherapy. s, the peak in rt o 5-FU and of 5-BU was observed.

Figure 1 - Representative chromatogram in HPLC of the white, in UV detector - 254 nm



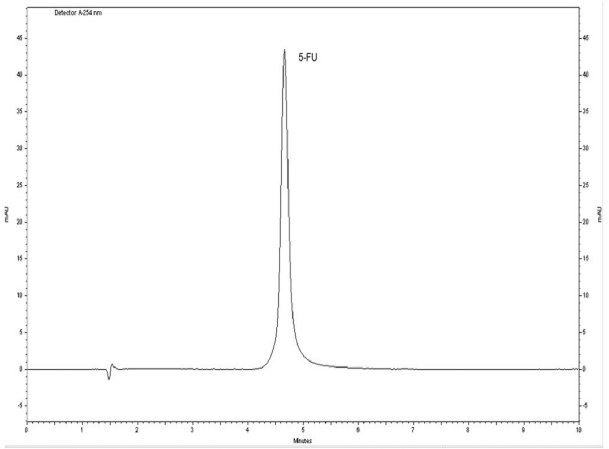
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 2 - Representative chromatogram, in HPLC, of an internal standard of 5-BU with 5 µg/ml, in UV detector - 254 nm



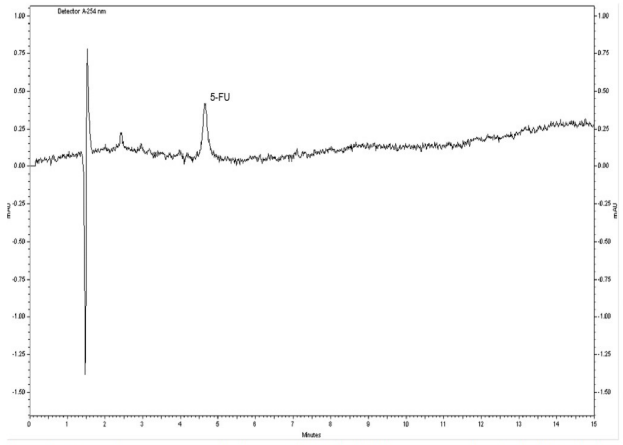
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 3 - Representative HPLC chromatogram of a 5-FU solution, with a concentration of 10,000 ng/ml, in a UV detector - 254 nm



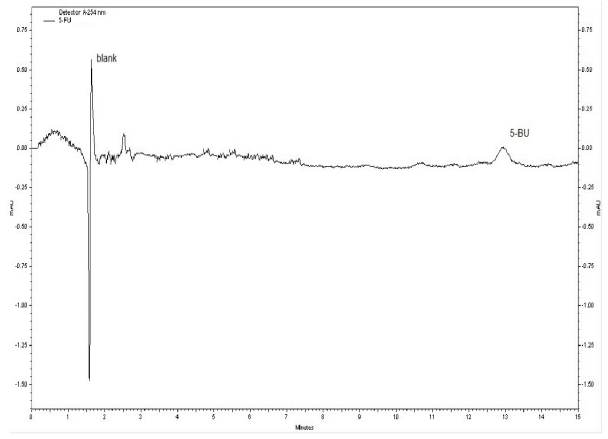
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 4 - Representative chromatogram, in HPLC, of 5-FU, with a concentration of 100 ng/ml, in a UV detector - 254 nm



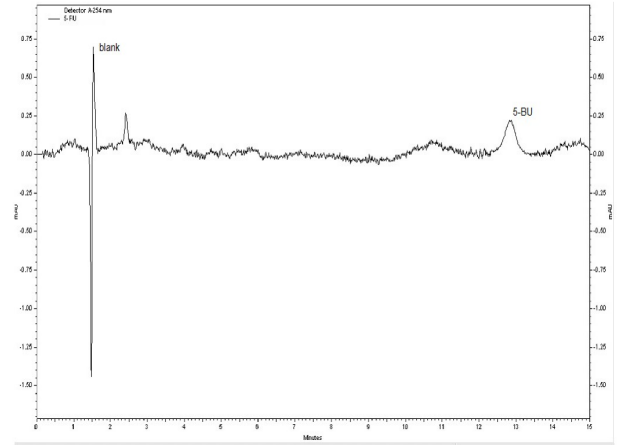
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 5 - Representative chromatogram, in HPLC, of a sample from the CP group, in a UV detector - 254 nm



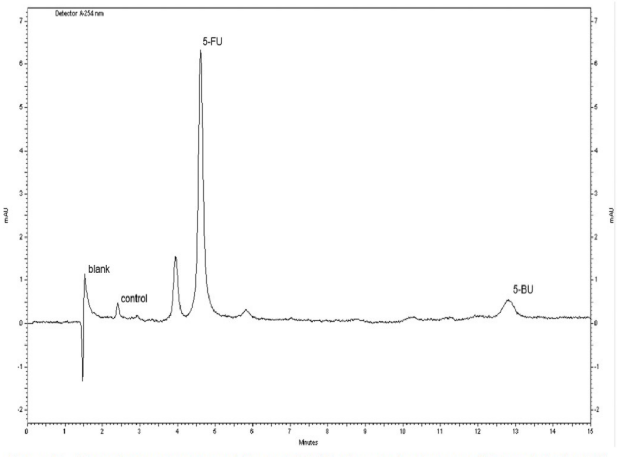
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 6 - Representative chromatogram, in HPLC, of a sample from the CPI group, in a UV detector - 254 nm



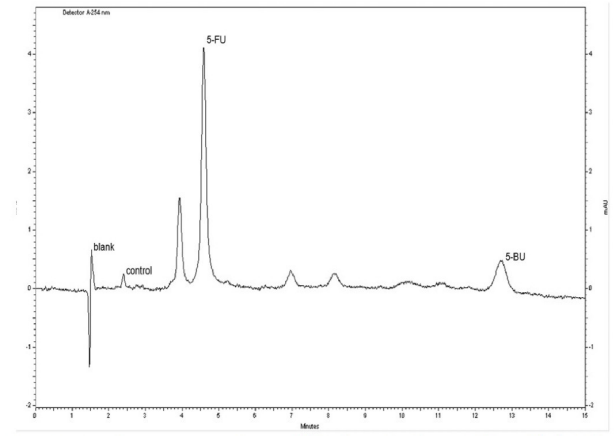
Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 7 - Representative chromatogram, in HPLC, of a sample from the QP group, in UV detector - 254 nm



Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Figure 8 - Representative chromatogram, in HPLC, of a sample from the QPI group, in UV detector - 254 nm



Source: High Performance Liquid Chromatography (HPLC) Shimadzu Prominence system (Shimadzu, Japan).

Some studies found in the literature have identified or quantified the important chemotherapeutic agent 5-Fluorouracil (5-FU), in samples of human[9,17], rat and dog fluids or tissues[1-4,18,19], by means of a model proposed by Sonis et al.[19] and later modified by other authors[10,11,20]. However, up to the time of the present research, the authors found no studies that determined the presence of this chemotherapeutic agent in the hamsters' saliva, animals widely used as a study model oral mucositis and salivary gland hypofunction, resulting from the intraperitoneal injection of 5-FU.

Since administration of the chemotherapeutic agent 5-FU alters salivary gland morphology and function, and the direct presence of the chemotherapeutic agent in the oral mucosa may lead to some oral complications, such as mucositis[1,9,10], and as there is no standardization in the literature of the technique for detecting 5-FU in hamster's saliva, determining the drug in the saliva of these animals may - in future studies - help with understanding how these oral complications occur after administration of this chemotherapeutic agent.

The authors who quantified 5-FU in human's[9,17] and rat's

saliva[1,2,4], did so by mean of HPLC analytical methods. For a long time now HPLC has been the model of choice among researchers and industries for separating and quantifying substances. Celio et al.[1] using *Wistar* rats as animal model, demonstrated that 5-FU was excreted in detectable quantities in parotid saliva after intravenous administration, by using HPLC analysis.

The determination of drug concentrations in saliva has gained widespread acceptance in a variety of settings. Salivary concentrations of drugs have been employed for therapeutic drug monitoring and for calculation of pharmacokinetic variables. Therefore, using saliva instead of blood for pharmacokinetic investigations has obvious practical advantages, particularly in children. It is a non-invasive procedure which avoids venipuncture and is amenable for collection of multiple specimens. It has been suggested that saliva can serve as an alternative body fluid for pharmacokinetic studies of certain drugs[21].

Therefore, the analysis of the present study were performed by means of liquid chromatography, and according to the official monograph USP 38 NF 33, 2015. In accordance with the general chapter <621> of American Pharmacopoeia[22], and considering the availability of columns and tests performed with 5-FU (standard), the column that presented satisfactory results was the one measuring 150 x 4,6 mm, maintaining the length/particle size ratio recommended. Therefore, from previous tests it was observed that in the active ingredient retention time, there was presence of analytical sign in the white solution, which could compromise the drug identification , due to co-elution. With the purpose of separating these analytical signs, a change was made in the proportion of the mobile phase that was defined as 100% of phosphate buffer 0.05 M, with pH adjusted to 5.7 ± 0.1. Another point modified in the cited protocol was the concentration of the 5-FU standard, since the mentioned method used the standard preparation at the concentration of 10 µg/ml, and according to the literature, some methods use detection of the active ingredient in saliva in concentrations of ng/ml[1,6,7,18].

From the first observations of the side effects of chemotherapeutic treatment, Celio et al, raised the hypothesis, (until today not confirmed), that the excretion in the saliva exposes the upper gastro-intestinal mucosa to the drug, and could therefore, be one of the irritant and synergistic factors of the drugs internal mechanism in the mucositis formation[1,7].

Added to this, and in view of the results found[10], such as alteration in the activity of some enzymes and in the morphology of the salivary glands of hamsters that received 5-FU, it has become necessary to standardize the technique for detecting this chemotherapeutic agent in saliva, and afterwards, in the salivary glands. The aim of the above-mentioned discourse is to gain better understanding of these oral complications resulting from the injection of 5-FU; and whether direct contact of the chemotherapeutic agent with the mucosa could be another irritant and causal factor of mucositis.

Celio et al.[1] has correlated the cytological changes in the rats 'salivary glands that received sublethal doses of 5-FU, with changes in the salivary secretion process, which could justify the dry mouth symptoms that some patients report after chemotherapy treatment.

Therefore, even by changing the mentioned method, it was possible for the authors of the present study to identify the presence of 5-FU in saliva samples from hamsters that had received intraperitoneal chemotherapy. Therefore, since 5-FU has been identified, the next studies could correlate the presence of the chemotherapeutic agent with the side effects this drug causes in the gastro-intestinal mucosa.

4 Conclusion

Based on the conditions of this study and results obtained, the authors may conclude that it is possible to identify the chemotherapeutic agent 5-FU in the saliva of hamsters that received the drug intraperitoneally, by means of High Performance Liquid Chromatography. In addition, it was concluded that the method used is sensitive for the proposed purpose.

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References

1. Celio LA, DiGregorio GJ, Ruch E, Pace JN, Piraino AJ. 5-Fluorouracil concentrations in rat plasma, parotid saliva, and bile and protein binding in rat plasma. J Pharm Sci 1983;72(6):597-9. doi: 10.1002/jps.2600720605
2. Hayashi Y, Watanabe J, Iwamoto K, Ozeki S. Salivary excretion of 5-fluorouracil. II. Fluctuation of saliva/plasma concentration ratio and salivar clearance during a constant rate intravenous infusion in beagle dogs. J Pharmacobiodyn 1988;11(6):438-43. doi: 10.1248/bpb1978.11.438
3. Hayashi Y, Watanabe J, Ozeki S, Iwamoto K. Salivary excretion of 5-fluorouracil (5-FU). III. Non-linear kinetics of salivary excretion of 5-FU following bolus intravenous administration in rats. Chem Pharm Bull (Tokyo). 1988;36(11):4547-53. doi: 10.1248/cpb.36.4547
4. Hayashi Y, Watanabe J, Ozeki S. Salivary excretion of 5-fluorouracil (5-FU). IV. Dependency of saliva/plasma concentration ratio and salivary clearance on plasma concentration of 5-FU during constant-rate intravenous infusion in rats. J Pharmacobiodyn 1989;12(3):137-44. doi: 10.1248/bpb1978.12.137
5. Bressolle F, Jacquet JM, Galtier M, Jourdan J, Donadio D, Rossi JF. Doxorubicin and doxorubicinol plasma concentrations and excretion in parotid saliva. Cancer Chemother Pharmacol 1992;30(3):215-8. doi: 10.1007/BF00686315
6. Joulia JM, Pinguet F, Ychou M, Duffour J, Astre C, Bressolle F. Plasma and salivary pharmacokinetics of 5-fluorouracil (5-FU) in patients with metastatic colorectal cancer receiving 5-FU bolus plus continuous infusion with high-dose folinic

acid. Eur J Cancer 1999;35(2):296-301. doi: 10.1016/s0959-8049(98)00318-9

7. Milano G, Thyss A, Santini J, Frenay M, Francois E, Schneider M, Demard F. Salivary passage of 5-fluorouracil during continuous infusion. Cancer Chemother Pharmacol 1989;24(3):197-9. doi: 10.1007/BF00300243

8. Epstein JB, Tsang AH, Warkentin D, Ship JA. The role of salivary function in modulating chemotherapy-induced oropharyngeal mucositis: a review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002 Jul;94(1):39-44. DOI: 10.1067/moe.2002.126018

9. Kumagai A, Iijima S, Nomiya T, Furuya I, Ohashi Y, Tsunoda K, et al. A pilot study of the clinical evidence for the methodology for prevention of oral mucositis during cancer chemotherapy by measuring salivary excretion of 5-fluorouracil. BDJ Open 2018;4:17041. doi: 10.1038/s41405-018-0008-2.

10. Alsarra IA, Alarifi MN. Validated liquid chromatographic determination of 5-fluorouracil in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2004;804(2):435-9. doi: 10.1016/j.jchromb.2004.01.043

11. Heggie GD, Sommadossi JP, Cross DS, Huster WJ, Diasio RB. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res 198715;47(8):2203-6.

12. Barbieri T, Costa KC, Guerra LFC. Alternativas atuais na prevenção e tratamento da xerostomia decorrente dos tratamentos antineoplásicos. RGO 2020;68. doi: https://doi.org/10.1590/1981-86372020000163546

13. Martins CG, Wagner SC, Linden R. Individualização Farmacocinética das Doses de 5-Fluoruracil no Câncer Colorretal. Rev Bras Cancerol 2013;59(2):271-80. doi: https://DOI.ORG/10.32635/2176-9745.RBC.2013v59n2.535

14. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3(5):330-8. doi: 10.1038/nrc1074

15. Campos L, Nicolau J, Arana-Chavez VE, Simões A. Effect of laser phototherapy on enzymatic activity of salivary glands of hamsters treated with 5-Fluorouracil. Photochem Photobiol 2014;90(3):667-72. doi: 10.1111/php.12195

16. United States Pharmacopeia. Monography Fluouracil. USP 38 NF 33; 2015.

17. França CM, França CM, Núñez SC, Prates RA, Noborikawa E, Faria MR, Ribeiro MS. Low-intensity red laser on the prevention and treatment of induced-oral mucositis in hamsters. J Photochem Photobiol B 2009;94(1):25-31. doi: 10.1016/j.jphotobiol.2008.09.006

18. Watanabe J, Hayashi Y, Iwamoto K, Ozeki S. Salivary excretion of 5-fluorouracil. I. Fluctuation of the saliva/plasma concentration ratio and salivary clearance in beagle dogs following bolus intravenous administration. Chem Pharm Bull (Tokyo) 1985;33(3):1187-94. doi: 10.1248/cpb.33.1187

19. Sonis ST, Tracey C, Shklar G, Jenson J, Florine D. An animal model for mucositis induced by cancer chemotherapy. Oral Surg Oral Med Oral Pathol 1990;69(4):437-43. doi: 10.1016/0030-4220(90)90376-4

20. United States Pharmacopeia. General chapter <621> Cromatography. USP 38 NF 33; 2015.

21. César IC, Cunha-Júnior GF, Duarte Byrro RM, Vaz Coelho LG, Pianetti GA. A rapid HPLC-ESI-MS/MS method for determination of dihydrouracil/uracil ratio in plasma: evaluation of toxicity to 5-flurouracil in patients with gastrointestinal cancer. Drug Monit 2012;34(1):59-66. doi: 10.1097/FTD.0b013e318240405f

22. Kumar AK, Sudha V, Srinivasan R, Ramachandran G. Simple and rapid liquid chromatography method for determination of moxifloxacin in saliva. J Chromatogr B Analyt Technol Biomed Life Sci 2011;879(30):3663-7. doi: 10.1016/j.jchromb.2011.09.047.