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Short communication

Potentiometric measurement of ionic, acid-labile and covalently bound fluorine

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Abstract

Fluoride (F^-) is the predominant chemical form of F in serum and bone, during administration of NaF as cariostatic agent or in the treatment of osteoporosis. In the treatment with sodium monofluorophosphate (MFP), F^-, F bound to proteins by acid-labile linkage and non-volatile covalently bound F are detected. Only F^- is detectable with the ion-selective electrode. This paper describes a method for the measurement of non-volatile covalently bound F with the ion-selective electrode, which has a detection limit of 0.8 ± 0.6 nmol, within-run standard deviation of 7 nmol and a between-run standard deviation of 13 nmol at 100 nmol F and has a linear behaviour above 1 nmol. This paper also reports a methodology for the potentiometric measurement of F^-, acid-labile F and covalently bound F in biological samples.

Keywords: Bone; Teeth; Fluoride; Fluorine; Fluorosis; Monofluorophosphate; Potentiometry

1. Introduction

Fluoride (F^-) is used as anti-caries agent [1] and as drug for the treatment of osteoporosis [2–4]. In all cases assessment of F^- concentration is a necessity, especially plasmatic levels in human beings, to avoid undesirable side effects [5]. Although instructions for the ion-selective electrode are clear and easy to follow, the F^- selective electrode used through the direct method can be employed only in aqueous solutions, and when fluorine (F) is present as F^- [6]. Potentiometric measurement of F^- in blood, which faces low concentrations and the presence of high levels of proteins, has been resolved with standards dissolved in plasma [7], with the addition–dilution method [8] or by mathematical corrections [9]. Few information is available about F in bone, especially because there is not a convenient technique that detects potentiometrically F containing compounds.

In the treatment with sodium monofluorophosphate (MFP), there is in plasma F bound to proteins by acid-labile linkage, apart from F^- [10]. We developed a method for the measurement of both compounds in biological samples [11]. Nevertheless, in bones of rats treated with MFP, apart from F^- as fluoroapatite, we have detected proteins with acid-labile F and non-volatile covalently bound F [12,13]. With the exception of F^-, these compounds are not detected with the ion-selective electrode by the direct method. This paper describes a potentiometric method for the measurement of non-volatile covalently bound F. It combines absorption of liquid samples on $CaSO_4$ tablets, incineration at 550 °C, isothermal distillation and measurement of voltage with an ion-selective electrode. This paper also reports the use of the ion-selective electrode in combination with isothermal distillation and incineration to measure F^-, acid-labile F and covalently bound F in bone material. This procedure together with the methodology for F measurement in plasma, will be an useful tool for the study of metabolism of covalently bound F, which probably are the compounds responsible for the high bioavailability of F in the treatment with MFP [14].

2. Results

If F concentrations are higher than 1 nmol, calibration curve for the measurement of covalently bound F after incineration and isothermal distillation was fitted by a linear function $(\text{mV}) = a + b \log(\text{nmol F})$, where $a = 365 \pm 47$ mV and

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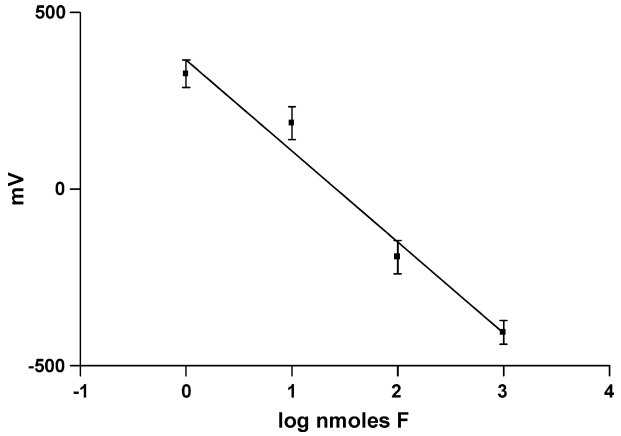

Fig. 1. Calibration curve for fluorine after incineration and isothermal distillation. Each point represents mean ± S.E.M. of four measurements.

$b = -257 \pm 27$ mV, $r = 0.9519$, $p < 0.0001$ (Fig. 1). When measuring a sample, linear regression is obtained with standards according to the concentration of samples. The curve was obtained with F concentration 1–1000 nmol, because biological samples are included in this range, but it can be constructed with higher amounts of F, when electrode is more sensitive.

Measurement of covalently bound F has a within-run standard deviation of 7 nmol at 100 nmol of F and a between-run standard deviation of 13 nmol at 100 nmol of F in samples. Within-run and between-run standard deviation increase dramatically when F is under 100 nmol, as a consequence it is recommended to measure samples with 100 or more nmol of F. The detection limit is 0.8 ± 0.6 nmol, independently of the volume of the sample. Reader should note that with this technique the volume of sample can be modified because it is added to a $CaSO_4$ tablet, which can be dried between aliquots of samples.

The F^- selective electrode employed in this work has a theoretical detection limit of 1 μmol/L (according to the instruction manual, Orion Research, Boston, MA, USA) in neutral solutions. The real detection limit of 0.1 μmol/L in distilled water with NaF standards, in absence of trivalent metals and when pH is in the range 5–8, was obtained with the methodology described in Section 4. In any other situation F^- measurement need additional procedures [7–9,11,15]. However, when F is bound by covalent linkage these methods do not detect the element. Gas chromatography [16] can be easily applied if compounds are volatile or can produce volatile derivatives. If these compounds are not possible to be obtained the mentioned technique is not useful. Another disadvantage is the high cost of the instrumental and the need of specially trained personnel.

2.1. Measurement of F in samples

The methodology (see Section 4 for details) was applied to EDTA extracts of bone material from rats treated with MFP for 30 days. F^-: 29.6 ± 2.5, acid-labile F: 8.0 ± 0.6 and covalently bound F: 96.3 ± 4.6. Results (mean ± S.E.) are expressed in μmol/g of dry bone. In the treatment with NaF, fluoroapatite is the most abundant compound in bone [17]. The described methodology was applied to EDTA extract of bone from NaF-treated rats. Results confirm that in bone of NaF-treated rats, F^- is the predominant chemical form of F. F^-: 84.8 ± 5.0, acid-labile F: 0.6 ± 0.4 and covalently bound F: 1.0 ± 1.4. In MFP-treated rats, bones contain F^- as fluoroapatite, acid-labile F bound to peptides and covalently bound F. The measurement of samples by direct potentiometry, potentiometry after isothermal distillation and potentiometry after incineration and isothermal distillation allows to measure F^-, acid-labile F and covalently bound F. This technique is useful for pharmacological studies where MFP is tested and bone composition must be analysed. The importance of compounds with non-volatile covalently bound F on bioavailability of MFP is still unknown, and experiments are being carried out to clarify this aspect.

As control of the technique, non-volatile covalently bound F content was measured in flurbiprophen tablets: 3958 ± 308. This value did not differ from the theoretical value: 4094 nmol F/mg flurbiprophen (one-sample Wilcoxon signed rank test $p > 0.05$).

3. Conclusions

The methodology proposed in this paper allows to measure ionic, acid-labile and covalently bound F in samples with the ion-selective electrode. The technique only requires a fluoride sensitive electrode, an incineration oven and disposable distillation chambers.

As liquid samples are deposited on $CaSO_4$ tablets, dried at 60 °C and incinerated at 550 °C this technique is not appropriate for samples where F is part of volatile compounds. As tablets are previously treated with NaOH, if compounds are destroyed by heat at temperature below boiling point, F^- is trapped in the tablet as NaF. On the contrary, F containing compounds escape from the tablet.

The method proposed in this paper, together with that developed for the measurement of fluorine compounds in blood, is an invaluable armamentarium for pharmacokinetic and pharmacological experiments, where MFP is used as osteogenic agent in experimental models of osteoporosis.

4. Experimental

4.1. F^- measurement by direct potentiometry

F^- was measured with an ion-selective electrode (94-09, Orion Research Inc., Cambridge, MA, USA). Electrodes were assembled as said by Hallsworth et al. to measure 20–50 μL samples [18]. Samples and NaF standards solutions was added 10% of total ionic strength adjustor buffer (TISAB III, Orion Research, Boston, MA, USA).

4.2. Samples and standards incineration

Liquid samples were absorbed on 50 mg F-free $CaSO_4$ tablet. Once the total volume was added to the tablet, they were

transferred to a porcelain crucible, and incinerated at 550 °C for 6 h. This process transforms F, independently of the chemical form, in NaF. Standard solutions of NaF containing 1000, 100, 10, and 1 nmol were processed in the same way as samples. The incinerated tablets were transferred to a distillation chamber and were subjected to isothermal distillation as stated below.

Tablets of $CaSO_4$ without samples or standard solutions were simultaneously subjected to the described procedure in order to assure that F containing compounds did not exist.

Preparation of $CaSO_4$ tablets: $CaSO_4 \cdot 1/2H_2O$ is mixed with enough distilled water to obtain a smooth paste and then it is deposited in moulds to obtain 50 mg tablets. $CaSO_4$ paste rapidly form a solid tablet. The absorption of liquid samples on $CaSO_4$ tablets has the benefit that after incineration the tablet with the sample is passed from the crucible to the distillation chamber easily with the aid of a forceps. If liquid samples with bone mineral are incinerated in porcelain crucible, after incineration the addition of acid and heat are needed to dissolve the ashes, and fluoride escapes as hydrofluoric acid.

Pre-treatment of tablets: they are previously treated with H_2SO_4 3 mol/L for a week (1 mL of solution per tablet) to eliminate acid-labile F as contaminant. Then, they are repeatedly washed with distilled water until pH is between 6 and 7, and then they are dried at 60 °C. After that, tablets were loaded with 20 μL of NaOH 1 mol/L and dried again at 60 °C. This addition assures alkali conditions that prevent the loss of F as HF in subsequent steps.

4.3. Isothermal distillation

As distillation chambers, 1.5 mL flat-bottomed polypropylene tubes were used. Twenty microlitres of 1.65 mol/L NaOH solution is deposited on the cap in small drops and acts as an alkali trap for HF [11]. After incineration, $CaSO_4$ tablets containing samples and standards are mixed with 400 μL of 3 mol/L H_2SO_4. The assembly is cupped and distillation of HF is allowed to proceed for 6 days at room temperature with constant mixing. HF is distilled into the alkali trap according to the Taves' procedure [15]. At the end of this period distillation was complete, the alkali trap was separated from the chamber, and a known amount (usually 20 μL) of 2.5 mol/L HOAc was added to the trap container to dissolve the residue (NaOH and NaF). The solution is adjusted to 100 μL with distilled water resulting a final pH 5.5. Finally F^- concentration is measured as stated above with the F^- selective electrode.

In order to assess the recovery of F^-, eight distillation chambers were loaded with 400 μL of 3 mol/L H_2SO_4, $CaSO_4$ tablets without samples or standards, and 20 μL of 1.65 mol/L NaOH solution were deposited in the cap. Chambers were subjected to distillation at room temperature for 6 days. At the end of this period they were opened and 1000, 100, 10 and 1 nmol NaF were added to the NaOH solution, it was then adjusted at pH 5.5 with 20 μL of HOAc 2.5 mol/L and finally adjusted to 100 μL with distilled water. The solutions were measured with the electrode as samples and standard.

Volume of samples, H_2SO_4 and NaOH can be modified in order to obtain better response of the electrode.

The addition of hexamethyldisiloxane (HMDS) to H_2SO_4 to shorten the period of distillation is a possibility. In the development of the technique HMDS was omitted because of it can cause skin irritation, its toxicology is not fully investigated, it is highly flammable, and time was not determinant in the study.

4.4. Reproducibility and detection limit

Tablets of flurbiprophene (a compound containing stoichiometric amounts of F bound by covalent non-volatile linkage) were subjected to the described methodology. Ten flurbiprophene tablets were crushed, solubilized in ethylic alcohol and aliquots of the solution were employed to load the tablets. Flurbiprophene contains 4094 nmol F/mg. Within-run standard deviation and between-run standard deviation were calculated with 100 nmol F from flurbiprophen [19].

Quadruplicates of 1000, 100, 10, 1, 0.1, 0.01 and 0.001 nmol of F from flurbiprophene were processed as stated above. The detection limit was determined from the point of intersection of the regression lines fitted to de Nernstian section of the F^- response curve (above 1 nmol) and the concentration-independent section of the curve (below 0.1 nmol) [20]

4.5. Methodology for the measurement of ionic, acid-labile and covalently bound F

Aqueous samples containing unknown chemical compounds with F must be subjected to the following procedure. F^- concentrations are measured in sample by direct potentiometry (*A*), direct potentiometry after isothermal distillation (*B*) and direct potentiometry after incineration and isothermal distillation (*C*). *A* accounts for F^- in the sample, *B* measures acid-labile F plus F^-, and *C* is the sum of F^-, acid-labile and covalently bound F. Different chemical forms of F can be calculated by applying the following equations:

$$F^- = A, \qquad \text{acid-labile F} = B - A,$$
$$\text{covalently bound F} = C - B$$

Aliquots of EDTA extracts of bone from MFP-treated rats ($n = 6$) and NaF-treated ($n = 6$) rats were subjected to the described methodology. Twenty-one-day old rats were treated with 80 μmol F/day in 1 mL of aqueous solution by gastric intubation for 30 days. After that, rats were euthanized with ethylic ether and femora were obtained. Femora were dried at 60 °C, weighted and cut with a scissors, and then they were ground in a mill. Two hundred milligrams of each sample were incubated at 4 °C with 5 mL of EDTA 0.5 mol/L pH 8, until bone mineral was completely extracted. Calcium, phosphate and fluoride concentrations increase following a constant ratio, as demineralization progresses [21]. The process was controlled by direct potentiometric measurement of F^- in the solution. Constant concentration of these ions indicates completeness of the process of demineralization. The process not only extracts bone mineral but soluble noncollagenous bone proteins as well. At the end of the process that takes as long as 4

days, bone is separated in insoluble collagenous matrix and EDTA solution containing bone mineral and noncollagenous bone proteins. The amount of different chemical forms of F was expressed in μmol/g dry bone.

4.6. Statistical techniques

Data are expressed as mean ± S.E.M. Comparison of one sample with theoretical value was performed using one sample Wilcoxon signed rank test, and regression lines were calculated using the linear least-squares method. A confidential level of 95% was considered significant.

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