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Fluorimetric determination of theophylline in serum by inhibition of bovine alkaline phosphatase in AOT based water/in oil microemulsion

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Abstract

Theophylline is an effective bronchodilatator used in the treatment of asthma which requires frequent control because of its narrow therapeutic index. Over the past decade much attention has been dedicated to the peculiar properties of the inner water pools of AOT (sodium 2-bishexyl-ethyl sulfosuccinate) microemulsions as enzyme microreactors, yet few analytical applications of the latter have been reported. We developed an original assay based on the uncompetitive inhibition by theophylline of the reaction catalyzed by alkaline phosphatase from bovine liver (E.C. 3.1.3.1) of the ELF-97® fluorogenic substrate in borate buffer 20 mM (pH 8.6)/AOT/iso-octane-ethyl acetate (95:5) at a temperature of 37°C. Optimal activity of endogenous plasmatic aIkaline phosphatase isoenzymes $\approx$ pH 10.5, interfering activity of the serum are avoided.The assay is multiple point rate, monitoring the appearance of the photostable fluorescence emission of the reaction product (510–530 nm) out of the water pool. The influence of several parameters such as the amount of buffer (W°), the amount of alkaline phosphatase, sample volume (10–30 pi), optimal run time (1–7 min) and the use of phosphorylating acceptor (2A2MP) are discussed. The method was compared to HPLC–UV and TDx methods.

Keywords: Bronchodilator; AOT microemulsions; Photostable fluorscence emission

1. Introduction

Theophylline is a drug used in the treatment of asthma and chronic bronchitis which are pathological conditions affecting the trachea and bronchii to various extents resulting in the narrowing of the airways. These symptoms are greatly reduced by a continuous theophylline therapy [1]. The pharmacological properties of theophylline include a potent bronchodilatating effect. Physiological conditions such as heart failure, liver disease, infection and obesity are known to reduce theophylline elimination. Theophylline has a shortened half life in smokers and in children [2]. Reported values for therapeutic serum concentrations are comprised between 6–20 mg l^{-1}. Toxic effects are related to the drug concentrations in the plasma. The symptoms include

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vomiting, nausea, headache, nervousness, and irritability. For higher concentrations cardiac arythmias, cerebral hypoxia, and cardiorespiratory collapses are reported. Although a good relationship exists between serum theophylline concentration and clinical response, high concentrations can produce life threatening toxicity. Frequent measurements can help prevent potentially fatal toxicity. Clinical efficacy of theophylline would be improved by individualizing dosages so that specific serum target theophylline concentrations are maintained. The narrow therapeutic index and the variability of theophylline concentrations in individuals underline the need for a rapid easy-to-use device for monitoring plasmatic concentrations [3]. To be useful at the hospital level, a theophylline assay must be fast (analysis time shorter than 15–25 min), accurate, and show good reproducibility with a relative SD within the range of 6.9–9.4% [4].

There are numerous analytical methods for quantitating theophylline such as spectrophotometry [5], high pressure liquid chromatography (HPLC) [6,7], electrochromatography [8] and isotope dilution mass spectrometry [9]. Immunoassays have gained wide popularity in clinical monitoring because of automation capability, limited sample treatment and relatively short analysis time [10,11]. Several companies provide immunoassay procedures such as AXSYM® fluorescence polarisation immunoassays (FPIA) from Abbott Lab and the Syva EMIT® method (enzyme-multipiled immunoassay technique). Chemiluminescent immunoassays have been reported also [12]. Other methods for theophylline clinical monitoring rely on the uncompetitive inhibition of bovine liver alkaline phosphatase in the presence of an appropriate substrate and are based on spectrophotometric [13], chronoamperometric [14,15], or potentiometric detection [16]. Reversed micelles (RM) based on Aerosol OT (AOT) represent interesting light transparent (UV-vis) microstructures for enzymes through solubilization in the aqueous core. RM are spherical aggregates of surfactant molecules dispersed in an apolar solvent. The polar heads of the surfactant molecules face into the interior water pool, whereas the hydrophobic tails are directed towards the bulk solvent. It is well established that proteins can be incorporated into RM while retaining their catalytic activity [17]. These microemulsions have been used in biotechnology for the extraction of proteins [18,19] and for getting better insight into the change of the tertiary structure of an enzyme entrapped in such biomimetic assemblies [20,21]. Potential advantages of these microstructures include a superactivity of solubilized enzyme mainly ascribed to the high concentration of the hydrophilic substrate in the enzyme surroundings [22]. Alkaline phosphatase has been reported to retain its catalytic properties in such hydrated surfactant aggregates [23]. This work has led to the development of a new analytical concept based on measuring enzymatic activity in reversed micelles [24] and exploiting the extraction and Actually quantitation of the enzymatic product in the organic phase. Actually, in this specific application, inhibition of enzyme activity by theophylline is quantified.

2. Material and methods

2.1. Reagents

AOT (bis-2-hexylethyl-sulfosuccinate), 2A2M-1P (2-amino-2-methyl-1-propanol), ALKP (alkaline phosphatase E.C. 3.1.3.1.), orthophosphoric monoester phosphohydrolase alkaline optimum (10 U mg^{-1}, pH 10.4) from bovine liver were purchased from Sigma MO. Iso-octane (analytical grade) and sodium tetraborate were obtained from Vel (Belgium), magnesium chloride ($MgCI_2 \cdot 6H_2O$) was obtained from Carlo Erba (Italy).

The ELF-97® phosphatase substrate and ELF-97® alcohol were available from molecular probes (Netherlands) as a 5 mM solution in sterile water and 1 mM solution in DMSO, respectively. Theophylline (Sigma) 400 mg l^{-1} stock solution was prepared with double distilled water. Borate buffer (20 mM) was prepared in double distilled water and the pH was adjusted to 8 6 with HCl (Merck). Phosphate buffer (10 mM) pH 4.4 was made by dissolution of KH_2PO_4 and adjusting the

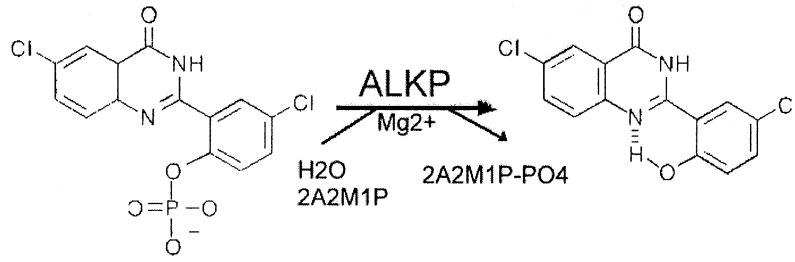


Fig. 1. Schematic mechanism of the ELF97® hydrolysis catalyzed by bovine alkaline phosphatase (ALKP).

pH with phosphoric acid (Merck). β-OH ethyltheophylline was purchased from Sigma. Human serum samples were obtained from patients monitored for theophylline by courtesy of the Algemeen Ziekenhuis-Vrije Universiteit Brussei-Klinische Chimie Department. Control sera Lyphocheck® (Bio-Rad) containing 25 drugs along with theophylline were used to check the accuracy of the method. Pooled serum was collected from healthy drug-free donors. The standards were prepared by supplementing portions of the pooled serum with the 400 mg l^{-1} theophylline stock solution.

3. Analytical procedures

HPLC–UV measurements were performed on a HP series liquid chromatograph using a Waters RP C18, 39 × 150 mm column. The mobile phase consisted of acetonitrile/phosphate buffer (94:6 v/v). The flow rate was 1.6 ml min^{-1}. The detector wavelength was set at 267 nm and the temperature of the column maintained at 50°C. The volume of the injection loop was 20 μl. Chromatogram were recorded using a Spectra Physics integrator. The samples were pretreated before injection. First 100 μl of internal standard (β-OH ethyl-theophylline 40 mg l^{-1}) were added to 100 μl of serum, then 2 ml of a chloroform/isopropanol (23:1 v/v) mixture was added and the resulting sample mixed using a vortex for 30 s (3 × 10). After centrifugation, the organic phase was dried under nitrogen. Finally the extract was dissolved in 100 μl of mobile phase. For FPIA 100 μl of plasma was pipetted into the sample well of an Axsym® instrument. The theophylline assay kit was used. Calibration of the FPIA instrument was achieved following manufacturer guidelines and the precision was checked daily using serum controls (low, medium, high) supplied by the manufacturer. Fluorescence assays were performed using a SLM Aminco 8000 C spectrofluorometer equipped with a Xenon Lamp (450 W). Quartz cells of 10 mm optical path were used. Unless otherwise stated the emission and excitation slits were both set at 16 nm. Spectra and kinetic curves were performed at 37 ± 1°C and recorded on a AT-80/286 GS-computer using the SLM Aminco aquisition software.

4. Results and discussion

The potential of reversed micelles to retain enzymatic activity of a solubilized protein in their aqueous core was exploited for the assay of theophylline in serum.

The reaction involved the cleavage of the phosphate group of the ElF-97® substrate to its corresponding alcohol (Fig. 1). This substrate was designed for ALKP activity detection [25]. Since the ELF -97® substrate is hydrophillic and negatively charged in our experimental conditions, it was assumed to be located exclusively in the water pool [26]. ELF-97® substrate is water soluble and not fluorescent in aqueous media, but after enzymatic hydrolysis, the liberated ELF-97® alcohol is water insoluble [27] and highly fluorescent in organic solvents due to a proton transfer from the phenol to the nitrogen resulting in a more rigid molecular structure [28]. Actually, the ELF-97® alcohol exhibited affinities towards both the surfactant layer and the organic phase (see below).

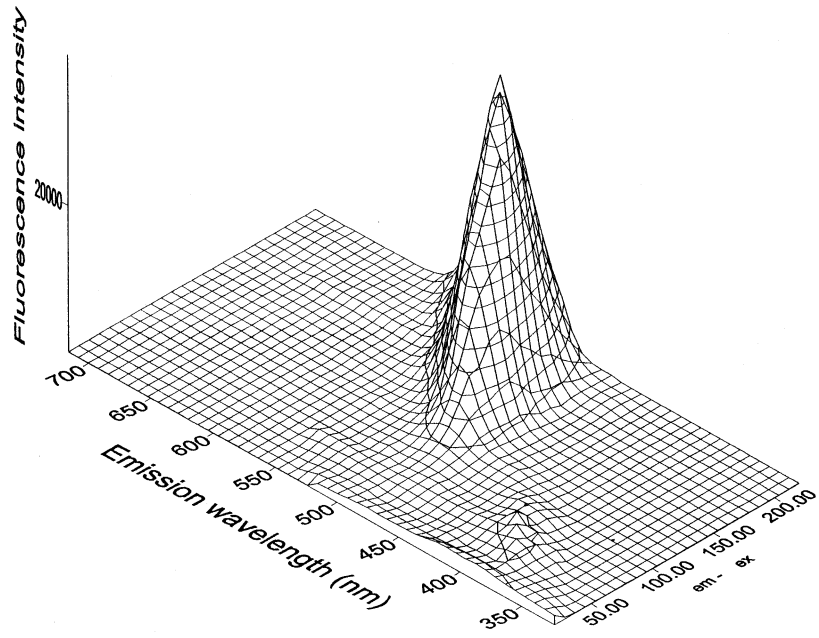


Fig. 2. 3D plot of fluorescence of ELF97® alcohol in borate buffer 20 mM, 2A2M1P 20 mM (W°16)/AOT 50 mM, iso-octane/ethyl acetate (95:5). Fluorescence intensity against emission wavelength and $\lambda_{em} - \lambda_{ex}$ (nm).

The extent of the interaction of the ELF-97® alcohol with the polar sulfonate group of the micelle constituting surfactants can be decreased by the use of a small amount of ethyl acetate. Indeed the latter was found to promote extraction of ELF-97® alcohol out of the water pool (see below) and so to develop an intense green fluorescence (Fig. 2). Basically, it was expected that the elimination of the enzymatic reaction product from the water pool will accelerate the enzymatic reaction as compared to a substrate yielding a hydrosoluble product which remains in the vicinity of the enzyme.

Fig. 2 indicates that the selected ELF-alcohol showed a broad fluorescence band located around 510–530 nm. Interestingly, at these wavelengths, the intrinsic fluorescence of the serum is negligible [29]. A hydrophobic fluorophore may adversely bind to serum proteins but the use of a w/o microemulsive system containing a high amount of surfactants and an apolar solvent minimized this effect, offering a substantial improvement of the fluorescence emission intensity: observed fluctuations were $< 6\%$ ($n = 14$).

5. Optimisation of the alkaline phosphatase activity in sodium 2-bishexyl-ethyl sulfosuccinate microemulsions

5.0.1. Magnesium and zinc

There are many choices for substrate and buffer but in most of the assays dealing with alkaline phosphatase or measuring alkaline phosphatase activity, magnesium is added to the reaction mixture. It is well known that this cation activates alkaline phosphatase and that optimal magnesium concentrations, when used in combination with alkaline phosphatase, lie between 3–5 mM [30]. Although Zn^{2+} can produce up to a 5-fold activity enhancement, the addition of this cation in the reactive buffer is questionable due to a concentration depending inhibition effect on the enzyme [31]. Magnesium is known to greatly stimulate

ALKP activity but this cation was also found to be responsible for nonlinearity of the initial kinetic plot [30]. Initial fluorescence measurements were therefore discarded for at least 80 s.

5.0.2. Buffer

A 20 mM borate buffer pH 8.4 may be suitable for alkalinization of the water pool of the microemulsion [23]. The optimal pH for human alkaline isoenzymes, although depending on the substrate concentration, is found between 10.4 for the liver ALKP isoenzyme and 10.7 for the placental ALKP [32]. For the selected pH 8.4, no interference from human ALKP was indeed observed, and bovine ALKP retained 70% of its activity with respect to its optimal activity which was $\approx$ pH 9 [14]. From the literature data, 23 possible chemicals that could act as a buffer for alkaline phosphatase, in the presence of *p*-nitrophenol-4-phosphate as substrate [5], it was obvious that a proper choice of the alkaline buffer was mandatory. Buffers containing one hydroxyl and a second hydroxyl group or an amino group allow faster removal of the phosphate from the enzyme active site than pure water. Buffers such as 2-ethylamino ethanol (2-EAE), diethanolamine or 2-amino-2-methyl-1-propanol (2A2M1P) exhibited the highest reaction rate in agreement with the literature [23,33]. Further experiments were realized using a mixed buffer based on borate and 2A2M1P (20 mM) [23].

5.0.3. Substrate and enzyme

In order to sustain a maximal velocity in the initial part of the kinetic curve, for a time period at least ten times greater than the time interval required for the fluorescence measurements, the amount of enzyme and the concentration of substrate in the aqueous pool of the microemulsion were optimized. The amount of enzyme investigated was in the 0.3–35 μg assay^{-1}. For 35 μg of bovine ALKP and a substrate concentration in the water pool of 300 μM, the reaction was completed after 3 min. In order to obtain a wider linear range, the amount of enzyme was decreased. For 0.3 μg assay^{-1}, a broad linear range was indeed obtained, but the kinetic curves exhibited poor sensitivity towards theophylline because of the slow reaction speed. A good compromise was achieved using 0.3 μg assay^{-1} of enzyme in the presence of 750 μM substrate in the water pool. These settings maintained the linear range for more than 15 min with a good sensitivity (see below). The overall concentration of substrate in the microemulsion was 11 μM that represented an aqueous concentration of substrate $\approx$ 750 μM.

5.0.4. Ethyl acetate

The nature of the organic solvent used for the formation of reversed micelles is a critical factor affecting the biocatalysis of entrapped enzymes [34]. Organic solvents with log *P* values > 2 are preferable since they offer good micelle stability and less risks of enzyme dehydration (log *P* octanol/water for iso-octane > 3.5, for ethyl acetate $= 0.66$) [35]. The initial step of the formation of an aqueous pool from an AOT micellar solution in iso-octane involves the hydration of the sulfonate groups of AOT. Expressing the water content by the molar ratio W° (water/AOT), the hydration of the surfactant was completed for W° between 8 and 10 in agreement with the literature [38]. A further addition of the aqueous buffer was required to ensure the formation of a bulk water pool in which enzyme solubilization can occur [36]. Curve A Fig. 3a, shows the fluorescence of the ELF-97® alcohol in a micellar solution of AOT in iso-octane without ethyl acetate. By addition of a small amount of water (W° between 1 and 2) (curve B Fig. 3a) an increase in the emission took place which was related to an increase in the microviscosity of the micellar inner interface [39]. For W° between 3 and 5 (curves C, D, E, Fig. 3a), the sharp decrease in fluorescence of the ELF-97® alcohol proceeded simultaneously with the hydration of the sulfonate group. The flat profile of the emission spectrum observed when the hydration process of the sulfonate was completed (W° > 8) [37] suggested that the fluorescence located around 510–530 nm was quenched (curve F, Fig. 3a). From these data, we may assume that the hydrated sulfonate heads interact with the ELF-97® alcohol inhibiting the internal molecular proton transfer i.e. resulting in the quenching of the fluorescence. The inferred interaction between the phenol and the anionic

surfactant underline that the ELF-97® alcohol may preferentially be located at the interface. Fig. 3b indicates that the extent of this interaction was greatly reduced by addition of 5% of ethyl acetate as observed by the sharply increased ELF-97® alcohol fluorescence. The fluorescence was found to be stable between 4 and 10% of ethyl acetate in iso-octane. This signal recovering in the presence of EA can likely be attributed to the penetration of the ELF-97® alcohol into the apolar side of the interfacial surfactant layer formed by the bulky hydrophobic tails. These assumptions were further supported by FTIR spectroscopy and FT-Raman experiments [40].

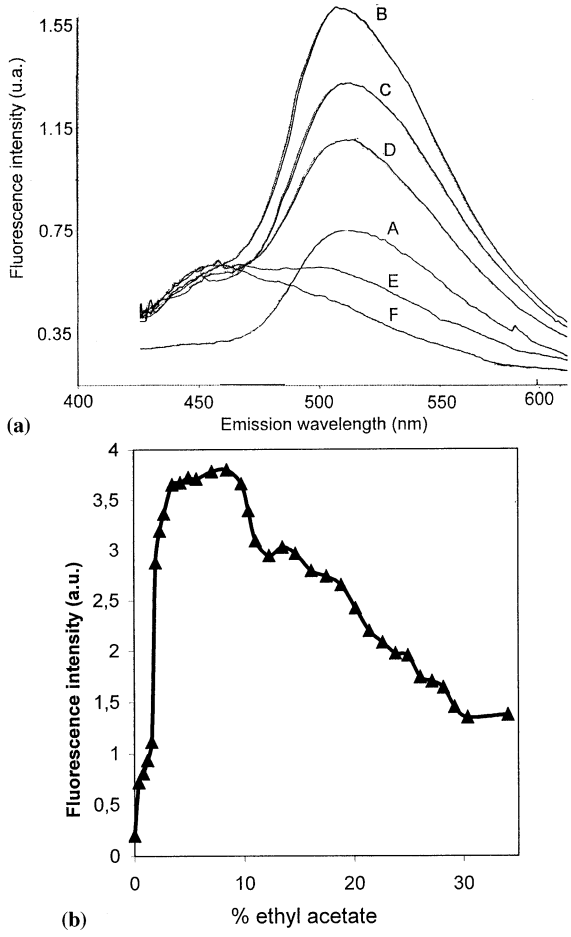


Fig. 3. (a) Effect of hydration (W°) on the fluorescence emission intensity of the ELF97® in AOT 50 mM/iso-octane, in the absence of ethyl acetate. A, B, C, D, E, and F, for W° = 0, 2, 3, 4, 5, and 8. Hydration performed with water; (b) Effect of ethyl acetate on the fluorescence of ELF97® alcohol in borate buffer 20 mM pH 8.5 (W° = 16)/AOT 50 mM/iso-octane.

5.0.5. Theophylline partitioning

Solubility experiments were performed in the absence of AOT and checking the partitioning by the described HPLC procedure (chromatograms not shown). Results indicated that theophylline protonated and molecular forms (pKa 8.8) were not soluble in iso-octane/ethyl acetate 95:5 from a 20 mM borate buffer solution (pH 8.4). The presence of theophylline was detected in the organic phase only for iso-octane/ethyl acetate ratios $<$ 60:40 v/v. Thus under our experimental conditions, the entire amount of theophylline, along with the substrate ELF-97®, were present in the aqueous core of the microemulsion.

5.0.6. Reagent sequence

From the above data, the borate-2A2M1P buffer pH 8.4 (W° = 16)/Aerosol OT 50 mM/iso-octane 95%/ethyl acetate 5% was selected. The entrapment of the enzyme into the reversed micelles was performed following the injection method [38] by addition of an aqueous solution of the enzyme to the organic solvent containing the surfactant. The measured values of enzymatic activity did not depend on the entrapment time, i.e. the activity of the entrapped enzyme, as inferred from the slope of initial velocity (see Fig. 4) remained unchanged for at least 6 h. Taking into account the uncompetitive nature of the inhibition [41], it was mandatory to apply the appropriate reagent sequence as follows: 20 μl of serum was mixed with 2500 μl of AOT/iso-octane/EA microemulsion W° = 16 (borate 20 mM/2A2M1P 20 mM/Mg^{2+} 3 mM) containing 11 μM of substrate. After thermostatization in a sealed quartz cell, the transparency was achieved within 4 min. The reaction was initiated by injecting to this microemulsion 200 μl of the AOT 50 mM/iso-octane/EA microemulsion W°16 (borate/2A2M1P/Mg^{2+} 3 mM) containing 3 pg 200 μl^{-1} of bovine ALKP.

5.0.7. Fluorescence measurements

Typical kinetic curves are illustrated in Fig. 5. Quantitative measurements were made after 80 and 200 s. The reaction velocity (V) was estimated

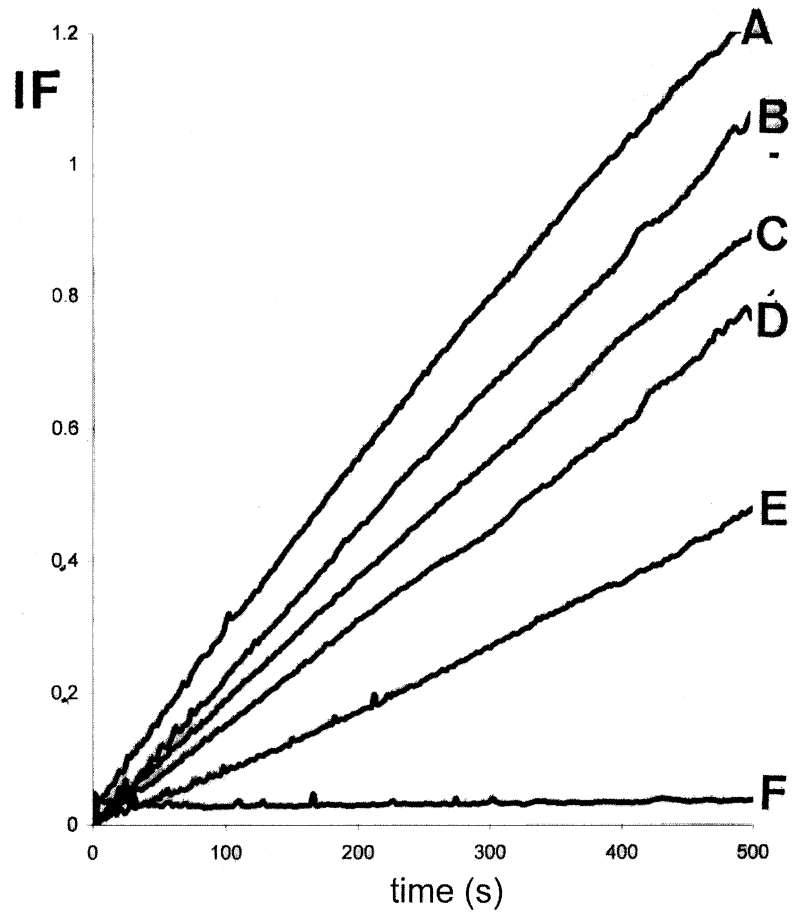


Fig. 4. Typical kinetic curves for serum calibration. AOT 50 mM/iso-octane/ethyl acetate (95:5). A, B, C, D, and E, for serum theophylline concentrations of 0, 4, 10, 20, and 40 mg l^{-1}, respectively. Blank run for the sample microemulsion without addition of exogenous bovine ALKP (curve F).

by the rate of fluorescence change. For the selected time intervals the concentration of substrate was still within 90% of the initial substrate concentration. The rate of the fluorescence change was then converted into enzymatic activity. Fig. 5 shows the relationship between V (Fig. 5a), 1/V (Fig. 5b) and the theophylline concentration in the serum. As expected, the latter yielded a straight line. The Dixon plot 1/V vs. inhibitor concentration in the aqueous pool enabled us to calculate the apparent inhibition constant K_i of theophylline: 78 ± 8 μM. This value is in good agreement with the K_i reported by Dai and Snow [41], 99 ± 28 μM . It is worth mentioning that Farley et al. [42], reported a $K_i <$ range for theophylline sensitive human alkaline phosphatase isoenzyme was between 0.15 and 0.2 mM . The K_i value of theophylline was pH independent between 8.7 and 10.1.

6. Application to serum samples and control serums

The proposed experimental setup for serum theophylline determination was compared with two other independent methods. HPLC–UV was considered as the reference method as it has a well established reliability [43]. In order to meet the requirements for clinical theophylline monitoring

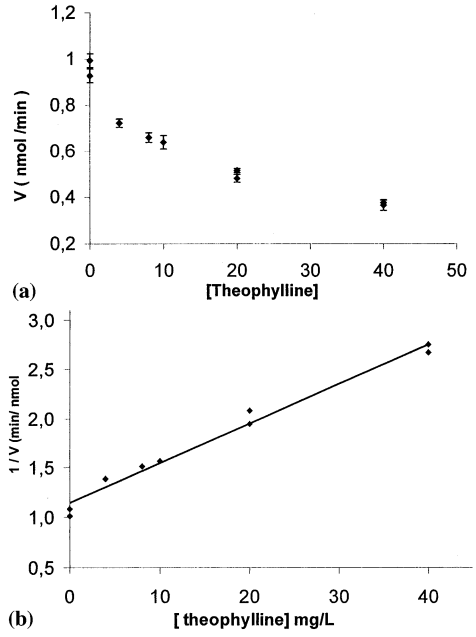


Fig. 5. (a) Plot of the ALKP activity (n mol min^{-1}) against theophylline serum concentration (mg l^{-1}); (b) Dixon plot of 1/ALKP activity against theophylline serum concentration.

we determined by FPIA, the average theophylline concentration range in serum from a clinical therapeutic monitoring trial over 3 months. The mean value obtained was 11 ± 6 mg l^{-1} ($n = 146$). A typical chromatogram obtained by the HPLC–UV reference method from a patient serum is shown in Fig. 6. Detection and quantification

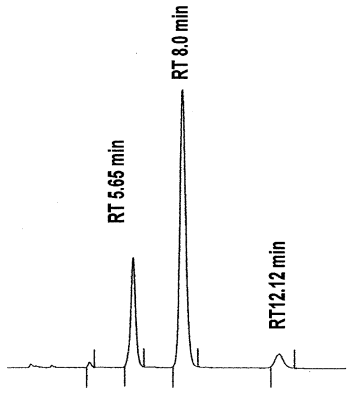
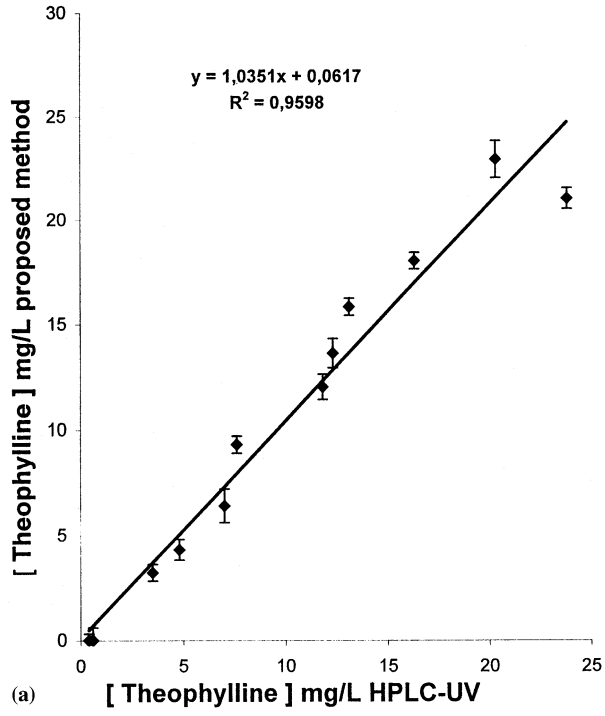


Fig. 6. Typical HPLC–UV chromatogram of human serum. Theobromine (RT: 3.99), theophylline (RT: 5.65 min), caffeine (RT: 12.12 min) and β-OH theophylline (IS) (RT: 8.0 min).

limits were calculated from k times the SD of the reaction speed measured in the 80–200 s range using $k = 3.3$ and 10, respectively. These were found to be 0.3 and 1.1 mg l^{-1}, respectively. The quantification range was comprised of 1–40 mg l^{-1}. Although rarely encountered. theophylline concentration > 40 mg l^{-1} required, a 1:2-fold dilution of the serum sample using BSA. A summary of the results on 12 serum samples is given in Table 1. The results obtained by the proposed method (microemulsion fluorescence enzyme inhibition: MFEI) were in good agreement with the results obtained by the reference methods (see Fig. 7a, b). In-between-day variation was estimated using the control serum over a five day period using different lots of microemulsions each day for three theophylline concentration levels. Reproducibility (C.V.) were of 7.6, 3.4, and 3.6, for theophylline concentration of 5, 15, and 25 mg l^{-1}, respectively. Overestimated theophylline concentrations were found for control lyphopheck L2, L3, compared to the HPLC–UV or the FPIA methods (Table 2) but were still within the acceptable range (L2: 15–23 mg l^{-1}, L3: 31–47 mg l^{-1}) supplied by the manufacturer for alkaline phosphatase inhibition methods. The comparison between HPLC–UV and FPIA (Fig. 7 c) allowed to point out the existing variation between two standard techniques.

6.0.1. Interfering species and sources of bias

The results obtained with the control serums (Table 2) indicated that other drugs present in serum and requiring therapeutic monitoring offer relatively little interference. The higher values obtained by our method, mainly for the control L2 and L3, are likely due to the presence of salicylate (15–42 mg dl^{-1}) for which a cross reactivity was reported [13]. The range of results acceptation for these control serum was actually higher for the Ektachem® method (Eastman Kodak Co.) which was also based on bovine ALKP inhibition. Caffeine produced no interference from 2 to 15 mg l^{-1}. The K_i value of caffeine (≈ 18 mM) is not encountered in serum even for toxic concentrations (20-40 mg l^{-1}). High endogenous alkaline phosphatase (e-ALKP) activity is a possible bias. No interference from endogenous activtiy was



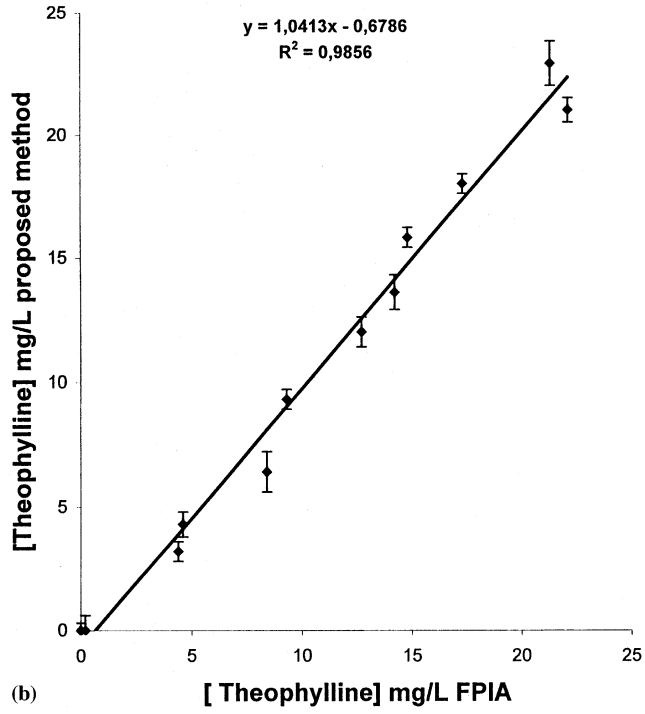
(a)

Fig. 7. (a) Comparison vs. HPLC–UV; (b) comparison vs. FPI; (c) intercomparison between both reference methods.

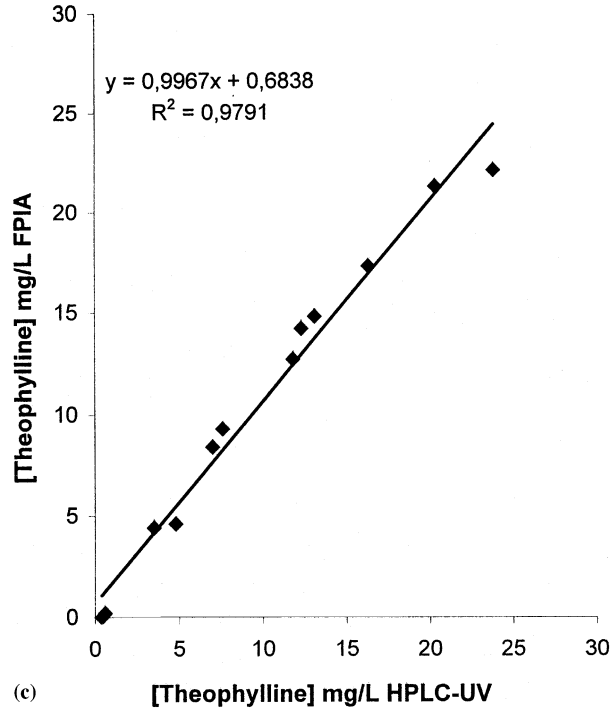
observed for normal serum e-ALKP activity (30–150 U l^{-1}). A pathological serum (e-ALKP of 600 U l^{-1}) produced a 6% negative bias for a theophylline concentration of 20 mg l^{-1}. Pathological states lowering renal clearance of theophylline metabolites, especially 3-methylxanthine and 1-methyluric acid, are possible positive bias. Although not tested, other uncompetitive inhibitors such as levamizole [44] and homoarginine at pathological levels (>100 μM) are potential interfering species.

7. Conclusion

AOT based water/oil microemulsions with entrapped alkaline phosphatase are suitable microstructures for the sensitive analysis of theophylline in a complex biological matrix. Advantages of the method rely on a narrow control of the distribution of the reactive species in the different phases of the microemulsion. A further possible development will involve decreasing the sample volume to less than 5 μl by reducing the quartz cell from 3 ml to 500 μl, successful results have already been obtained. From a practical point of view though, a reduction of the sample volume require automation capability in order to minimize sampling errors.The original developed method met the requirements for clinical theophylline monitoring at low cost, with an on board time of 10 min, a running time of 6 min and a sample volume of 20 μl. Recent developments of reversed microemulsion based membranes represent also new trends for solid phase microemulsion based optrodes [45].



(b)



(c)

Fig. 7. (*Continued*)

Table 1
Results of serum samples for the proposed and reference methods

Samples	Reference method A	Reference method B	Proposed method	
	HPLC–UV found ($n=1$)	FPIA found ($n=1$)	MFEI found ($n=3$)	
			Mean	SD
1	0.4	0.0	0	0.3
2	0.6	0.2	0	0.6
3	3.5	4.4	3.2	0.4
4	4.8	4.6	4.3	0.5
5	7.0	8.4	6.4	0.8
6	7.6	9.3	9.3	0.4
7	11.8	12.7	12.0	0.6
8	12.3	14.2	13.6	0.7
9	13.1	14.8	15.8	0.4
10	16.3	17.3	18	0.4
11	20.3	21.3	22.9	0.9
12	23.8	22.1	21.0	0.5

Table 2
Results of control serum for the proposed and reference methods

Sample ($n=5$)	HPLC–UV		FPIA		Proposed method	
	Mean	SD	Mean	SD	Mean	SD
L1	4.4	0.2	6.9	0.4	5.1	0.4
L2	14.0	0.3	11.9	0.6	14.8	0.5
L3	27.2	0.4	26.0	1.2	35.8	1.3
0.3						
0.6	4.8–7.1		6.3–7.7		4.5–6.5	
0.4	11.0–16.0	10.8–13.2	15.0–23.0			
0.5	21.0–32.0	23.4–28.6				31.0–47.0

Acknowledgements

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