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Laser induced resonance energy transfer – a novel approach towards achieving high sensitivity in capillary electrophoresis I. Clinical diagnostic application

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Abstract

Most of the procedures currently performed by capillary zone electrophoresis (CZE) with laser induced fluorescence detection requires prior derivatization. This increases cost, the turn-around-time and chances of extraneous contaminations. CZE with laser induced resonance energy transfer is demonstrated as a viable alternative for detecting non-fluorescent compounds with no prior derivatization. The feasibility of this approach is demonstrated by separating and directly detecting salicylic acid (2,4-dihydroxybenzoic acid), gentisic acid (*o*-methoxybenzoic acid), salicyluric acid (*o*-hydroxyhippuric acid) and 4-aminosalicylic acid in urine. The detection of salicylate in serum is also shown. The method is highly sensitive with detection limits in the $1 \cdot 10^{-7}$ *M* range. Importantly it requires no prior preconcentration or sample preparation and can be used with complex sample matrices such as serum and urine.

Keywords: Detection, electrophoresis; Laser-induced resonance energy transfer; Salicylic acid; Gentisic acid; Salicyluric acid; Aminosalicylic acid

1. Introduction

Capillary electrophoresis (CE) is beginning to be evaluated as a method to replace classical electrophoresis and high pressure liquid chromatography (HPLC) in clinical laboratories. The only aspect of CE holding it back from taking its place in clinical laboratories is the lack of sensitivity due to the extremely small volume of sample used in the analysis. Thus, improvement of sensitivity is an area of major emphasis in CE. Generally, three detection modes are used in commercial CE instruments, absorbance, fluorescence, and conductance [1–6]. Absorbance detectors are the most widely used and have the advantage of being relatively simple and universal but provide low sensitivity due to the short optical pathlength. Sensitivity of fluorescence, the second most widely used method, is directly proportional to the power of excitation source. Therefore sensitivity in femto molar range can be easily achieved by using high powered lasers in CE with laser induced fluorescence (LIF) detection [7]. Conductimetry detectors, although simple and universal, require periodic cleaning of the working electrode for optimum detector performance. In addition, the electrical isolation of the working electrode from the high potential field across the electrophoresis capillary can often be difficult. Of the routinely used methods of detection, LIF offers the highest sensitivity.

Although LIF is highly sensitive the analyte must be fluorescent or be made fluorescent by tagging it with a suitable fluorophore. In addition, there are only two lasers with appropriate emission lines, an

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argon ion (488 nm) and a helium–cadmium (HeCd) (325 nm) laser, readily available and affordable for use in CE. These lasers detect fluoresceinated and dansylated compounds. The procedures to derivatize a molecule with fluorescein or dansyl chloride are lengthy, not robust at low analyte concentrations, and increase the chances of extraneous contamination. Even with these shortcomings, LIF is increasingly being used to detect various biologically important analytes such as amino acids, proteins and nucleotides. A method capable of exploiting the high sensitivity of LIF, but requiring no derivatization would be indispensable in the development of routine assays on CE.

Resonance energy transfer (RET) is one way to achieve high sensitivity while avoiding derivatization. RET is the transfer of energy absorbed by a donor molecule to an acceptor molecule with subsequent fluorescence from the acceptor. It has been widely used in studies of bimolecular structure and dynamics [8]. An important aspect of this approach is that the analyte need not be fluorescent or be made fluorescent. Ideally, the acceptor molecule should be soluble in aqueous buffer, have minimal absorbance at the absorption maxima of the analyte, show fluorescence only in the presence of the analyte molecule, and be capable of accepting energy with high efficiency from a wide range of substances.

The lanthanides, in particular terbium (Tb) and europium (Eu), have the potential of fitting the criteria of an ideal acceptor. They can accept energy from a wide range of compounds, have minimal absorbance and intrinsic luminescence in aqueous solutions and exhibit several fold enhancement in ion fluorescence upon sensitization by a donor [9]. Lanthanide sensitization has been used in HPLC as the method of detection for a variety of compounds, e.g., aromatic ketones, tetracycline, nucleic acids and polynucleotides and several thiols derivatized with 4-maleimidylsalicylic acid [10–14]. However its application for direct detection in CE is relatively unexplored. Nielen first used Tb-acetylacetonate complex for indirect time resolved luminescent detection of anions such as nitrite, chromate, hexacyanoferrate, ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid and alkyl sulfates on CE [15]. More recently, Latva et al. described the construction of an on-column time resolved luminescence detector for CE and used it for characterizing europium chelates of heptadentate ligand [16]. However, in spite of RET application in this method, prior derivatization of the heptadentate ligand with a donor molecule was a requirement, making it similar to previous LIF procedures.

The feasibility of using CE with laser induced RET detection in the development of sensitive, yet simple, assays in the area of clinical chemistry is the crux of this paper. There have been an increasing number of clinical assays developed on CE [17–20]. However, one of the main problems encountered in developing clinically useful assays on CE is interference from naturally occurring UV absorbing substances such as proteins, uric acid, creatinine, etc. in serum and urine. Using the metabolites of aspirin in urine and serum we have for the first time demonstrated the advantages of laser induced RET in a clinical diagnostic application using CE. The most important aspect of this assay method is that no prior sample preparation or derivatization was required for serum or urine which are extremely complex sample matrices.

2. Experimental

2.1. Instrumentation

CE analysis was performed on a Beckman P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with System Gold software for data analysis. A LIF detector equipped with a 20 nm bandpass filter was used for monitoring terbium ion luminescence at 547 nm. A 325 nm HeCd Series 39 laser (Omnichrome, Chino, CA, USA) was used for excitation. The salicylic acid metabolites were separated in a 45 cm, injection to detection, fused-silica capillary. High pressure injection were made for 5 s and the temperature of the capillary was maintained at 16±0.1°C. The applied voltage was 20 kV and operating currents were less than 100 μA.

2.2. Materials and method

Sodium salt of salicylic acid (2,4-dihydroxybenzoic acid), gentisic acid (*o*-methoxybenzoic acid), salicyluric acid (*o*-hydroxyhippuric acid), 4-amino-

salicylic acid, sodium carbonate, EDTA (as disodium salt) and terbium chloride (TbCl, 99.99%) were purchased from Sigma (St. Louis, MO, USA). A run buffer composed of 0.002 *M* EDTA, 0.002 *M* $TbCl_3$ and 0.02 *M* Na_2CO_3 with pH adjusted to 11 by 0.1 *M* NaOH was used for resolving salicylic acid derivatives. The buffers were prepared once a week and filtered through 0.45 μm nylon syringe filters (Waters, Milford, MA, USA). Stock solutions (0.01 *M*) of salicylate and its metabolites were prepared by dissolving the respective compounds in deionized water and then diluting in deionized water to prepare working standards with concentrations of $1 \cdot 10^{-3}$, $5 \cdot 10^{-4}$, $1 \cdot 10^{-4}$, $5 \cdot 10^{-5}$, $1 \cdot 10^{-5}$ and $1 \cdot 10^{-6}$ *M*. Each of the standards were run in sets of five so as to monitor the precision of migration times for each analyte.

The fused-silica capillary was activated by rinsing it with 0.1 *M* NaOH for 30 min followed by distilled water for an equal period of time. Before each injection the capillary was equilibrated with run buffer for 3 min. At the end of each run the capillary was washed for 3 min with 0.1 *M* NaOH followed by deionized water.

Serum and urine samples were spiked with salicylate or its metabolites to give concentrations of $1 \cdot 10^{-4}$ *M* and were directly injected for 5 s without further sample preparation.

3. Results and discussion

Caslavska et al. first reported the use of CE to separate and quantitate salicylate and acetaminophen in three emergency room cases. The diode array detector used by these authors was able to detect salicylate only at relatively the high concentration of 3.73 m*M* [21]. As demonstrated by these authors, separation of salicylates by CE is trivial because of its high resolving power. However, detection of salicylate in urine and serum using absorbance detection is difficult due to the presence of numerous UV absorbing compounds in these body fluids. This was confirmed in the present study as indicated in Fig. 1a and b which shows the absorbance at 254 nm of an electropherogram of a typical blank urine and a urine spiked with $1 \cdot 10^{-4}$ *M* salicylates. As seen in the figure the many normally occurring endogenous constituents in urine make identification of salicylates impossible. In contrast the electropherograms (Fig. 2a and b) obtained when laser induced RET (salicylates to terbium) was used, monitoring Tb^{3+} luminescence at 547 nm. The electropherogram obtained by using RET is much simpler and shows no interference from other normally occurring components of urine when compared to the electropherogram obtained by using an absorbance detector. This is due to the inability of these components to transfer energy (sensitize) to Tb^{3+}. The slightly distorted peak for hippuric acid is due to the electrodispersion caused by differences in conductivity between the hippuric acid and running buffer. Similar electropherograms obtained from blank and serum spiked with salicylic acid ($1 \cdot 10^{-4}$ *M*) are shown in Fig. 3a and b. Thus, RET detection not only permits the sensitivity of laser induced fluorescence, but at the same time simplifies the resulting electropherogram considerably.

The most popular method for the measurement of salicylate in serum is Trinder's method (see Veltri and Thompson [22]). It is based on the reaction between salicylate and Fe^{3+} in acid to form a colored complex that is measured at 540 nm. Although easy to perform, the method is non-specific. There are interferences due to serum proteins, salicylic acid metabolites and structurally related drugs such as diflunisal (difluorophenyl salicylate) [23]. Other methods for salicylate quantitation include fluorescent polarization immunoassay [24] and a salicylate monoxygenase-mediated photometric method [25]. These procedures are also subject to similar interferences as the Trinder method. Gas (GLC) and liquid chromatographic (HPLC) methods are the most specific methods for salicylate quantitation and are usually considered as reference methods [26,27]. Although used for pharmacokinetic studies or therapeutic drug monitoring of salicylate, the chromatographic procedures are not readily amenable to biological samples such as urine and serum. Both HPLC and GLC require sample preparation to remove interference from endogenous proteins present in biological fluids.

Aspirin is rapidly absorbed from the gastrointestinal tract reaching a peak serum concentration within 2 h [28]. Once absorbed, aspirin has a very short half-life ($t_{1/2} \sim 15$ min) due to rapid hydrolysis to

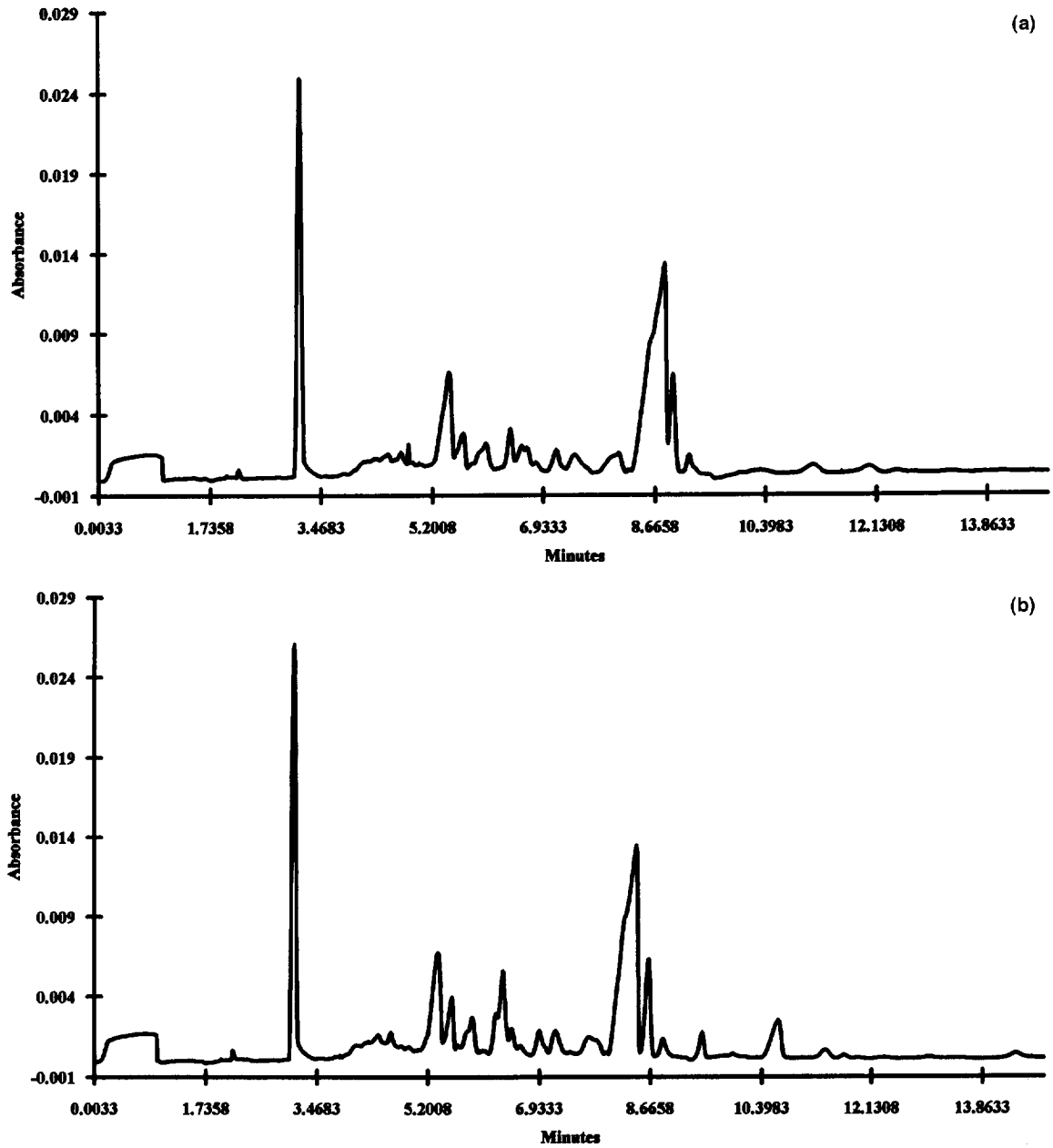

Fig. 1. (a) Blank urine, absorbance detection at 254 nm. (b) Spiked urine containing salicylic acid, gentisic acid, salicyluric acid and 4-aminosalicylic acid at $1 \cdot 10^{-3}$ *M* level, absorbance detection at 254 nm.

salicylate by the liver. Salicylate ($t_{1/2}$~2 h) is eliminated mainly by conjugation with glycine to form salicyluric acid, and to a lesser extent with glucuronic acid to form phenol and acyl glucuronides. A small amount is hydroxylated to gentisic acid. At high therapeutic doses these metabolic pathways are saturated. Consequently, serum salicylate concentration may increase disproportionately with dosage. At toxic doses, salicylate elimination half life is prolonged (15–30 h vs. 2 h at low dose),

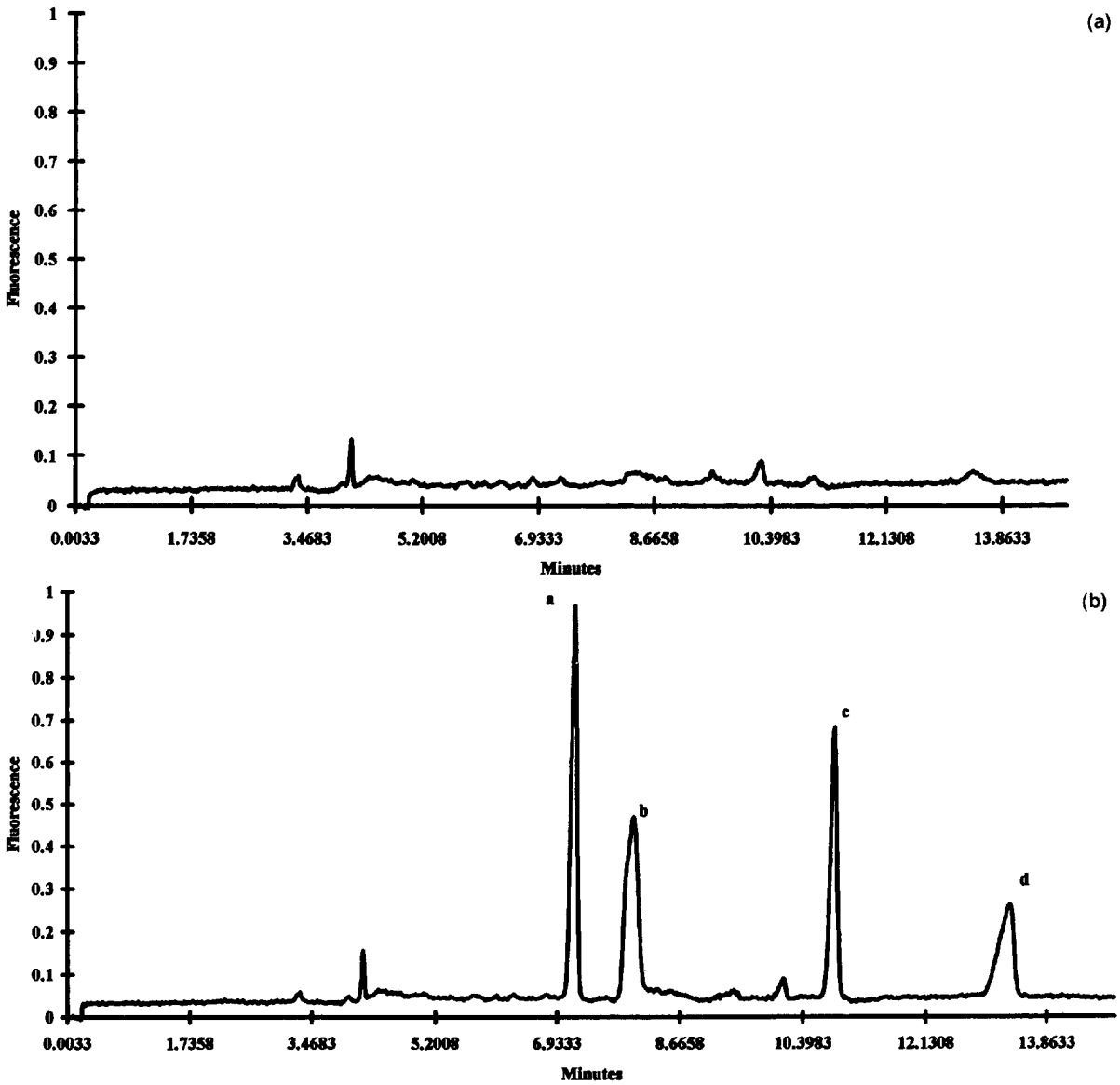

Fig. 2. (a) Blank urine, laser induced RET detection, λ_{ex} 325 nm and λ_{em} 547 nm. (b) Spiked urine containing (peak a) 4-aminosalicylic acid, (peak b) salicylic acid, (peak c) gentisic acid and (peak d) salicyluric acid at $1 \cdot 10^{-4}$ *M* level, laser induced RET detection, λ_{ex} 325 nm and λ_{em} 547 nm.

and a much larger portion of dose is excreted in urine as free salicylate [28].

3.1. Efficiency, sensitivity and linear range

Table 1 gives the detection limits and linear ranges obtained for salicylate and its derivatives. The corrected peak areas vs. concentration calibration curves were linear over three orders of magnitude with correlation coefficients ranging from 0.9532–0.9801. The sensitivity for salicylate ($1 \cdot 10^{-7}$ *M*) and its metabolites ($1 \cdot 10^{-6}$ *M*) could be improved by increasing the Tb-EDTA concentration. However, the increased ionic strength caused a considerable

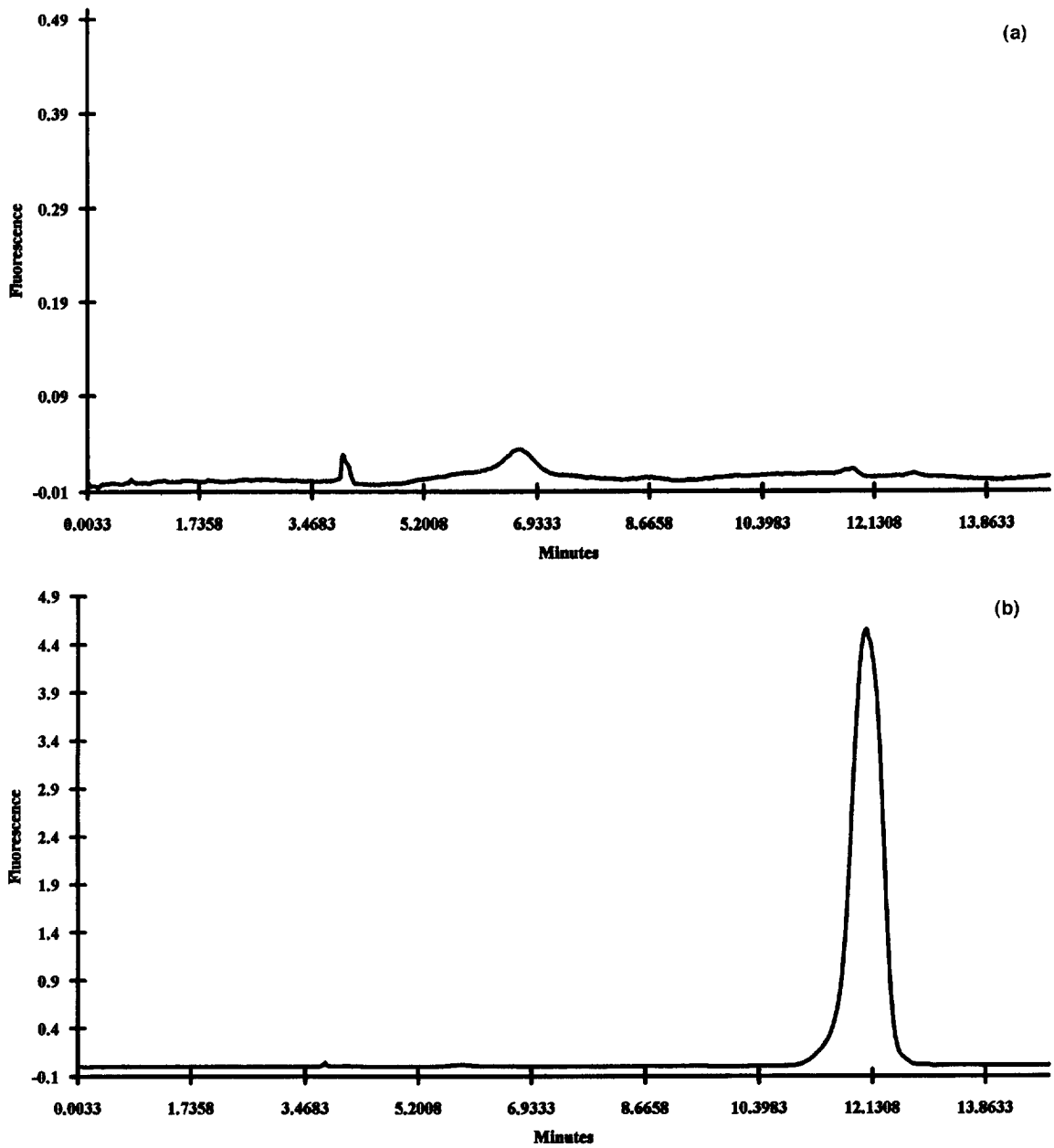

Fig. 3. (a) Blank serum, laser induced RET detection, λ_{ex} 325 nm and λ_{em} 547 nm. (b) Serum spiked with $1 \cdot 10^{-4}$ *M* salicylic acid, laser induced RET detection, λ_{ex} 325 nm and λ_{em} 547 nm.

increase in both current and migration times, thus limiting EDTA-Tb concentration to less than 0.01 *M*. Gentisic and hippuric acid could not be detected at the same levels as the other compounds probably due to the decreased molar absorptivity of these analytes at 325 nm. The percentage recovery calculated from the spiked urine samples ranged from 98–110% for salicylate and metabolites. The theoretical plates which ranged from 175 000 to 200 000 (Table 2) were calculated from the equation,

Table 1
Linear range and detection limits

Analyte	Linear Range	Detection Limit
4-Aminosalicylic acid	$1\cdot10^{-6}$ M–$1\cdot10^{-3}$ M	$1\cdot10^{-7}$ M
Salicylic Acid	$1\cdot10^{-6}$ M–$1\cdot10^{-3}$ M	$1\cdot10^{-7}$ M
Gentisic Acid	$1\cdot10^{-6}$ M–$1\cdot10^{-3}$ M	$1\cdot10^{-6}$ M
Hydroxyhippuric acid	$1\cdot10^{-6}$ M–$1\cdot10^{-3}$ M	$1\cdot10^{-6}$ M

$$\text{Theoretical plates} = 5.54[L_d/(\text{area}_{corr}/\text{peak height})]^2$$

where L_d is length to the detector and $area_{corr}$ is corrected area [29].

4. Conclusion

Using salicylate and its metabolites, we have shown that laser induced RET is a viable and simple mode of detection in CE. It offers the numerous advantages of no sample preparation for urine or serum, is highly sensitive ($\sim 1\cdot10^{-7}$ M), and has no interference from naturally occurring endogenous materials present in serum and urine. The use of the 325 nm HeCd laser used in this application does limit this technique to the analytes that absorb at this wavelength. Nevertheless, it proves that with a laser having the appropriate emission line, RET can be used as a general method for detection in CE. The lanthanides, Tb^{3+} and Eu^{3+}, are good acceptors being soluble in aqueous medium and exhibit several fold enhancement in ion fluorescence in the presence of donor compounds. In addition, they accept energy from a wide range of organic compounds and their long luminescence lifetimes permits use of time gated detectors (time-resolved luminescence) which has been shown to increase sensitivities in CE by increasing S/N ratio [16].

Using RET, the lanthanides, Tb^{3+} and Eu^{3+}, and CE there are many potential applications in the clinical laboratory. A pulsed or continuous laser with an emission line around 240 nm can potentially yield a relatively simple procedure for the determination of numerous clinically important analytes such as amino acids and organic acids. These analytes are extremely useful in helping the physician make the clinical diagnosis of various in-born errors of metabolism. The determination of anabolic and clinically relevant steroids (e.g. the steroids elevated in congenital adrenal hyperplasia) in serum and urine is another area where this concept can be applied to develop simple clinical assays. These steroids possessing an α,β-unsaturated carbonyl group have been shown to sensitize terbium efficiently in a sodium dodecyl sulfate micellar medium [30].

Table 2
Efficiences for different salicylates

Analyte	Theoretical plates = $5.54[L_d/(\text{area}_{corr}/\text{peak height})]^2$
4-Aminosalicylic acid	186 456
Salicylic Acid	180 234
Gentisic Acid	200 110
Hydroxyhippuric acid	175 300

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