Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 687–695, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00
Pergamon Press plc

Conventional and laser induced fluorescence detection of glucuronic acid conjugates after derivatization and liquid chromatographic separation*

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Abstract: Pre-chromatographic derivatisation of the carboxylic acid function of glucuronic acid conjugates is a suitable method for the selective and ultra-sensitive analysis of these compounds in urine and plasma samples. This goal is achieved by applying an indirect derivatisation procedure and laser induced fluorescence detection with a home-made detection system equipped with a continuous-wave argon-ion laser. The minimum detectable amounts for the analytes, after derivatisation, are about 3 amol using the fluorescein fluorophore. In comparison with conventional induced fluorescence detection a gain in sensitivity of over four orders of magnitude is obtained.

Keywords: *Liquid chromatography; conventional and laser induced fluorescence detection; glucuronic acid conjugates; derivatisation; plasma and urine samples.*

Introduction

Studying the pharmacology and the pharmacokinetics of drugs, the availability of ultra-sensitive and selective bioanalytical methods for these analytes (e.g. peptides, steroids, opiate alkaloids) and their metabolites (e.g. glucuronic acid conjugates) is of great importance. For instance in order to study *in vivo* intestinal glucuronidation processes [1].

However, the application of analytical methods based on the hydrolysis of the glucuronides, followed by the analysis of the parent compounds may result in overlooking important information on the metabolic routes of conjugation. Therefore, it is essential to analyse the intact glucuronides.

In order to fulfil the analytical prerequisites (selectivity and sensitivity), the combination of a liquid chromatographic (LC) separation step and a sensitive and selective detection method is one of the obvious choices for the bioanalysis of these solutes in the low pg ml^{-1} or even in the fg ml^{-1} range.

In spite of the variety of detection systems that can be applied in LC, the necessary

* Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

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sensitivity can only be obtained with a limited number of these techniques e.g. chemiluminescence, amperometric, and fluorescence methods. However, laser induced fluorescence detection seems to be the most suitable technique to achieve detection and determination limits in the low fg ml^{-1} range [2]. This high sensitivity is needed to study, for instance, the *in vivo* glucuronidation processes of xenobiotics in the intestinal wall or the intestinal epithelial cells of the rat [3, 4]. In this case plasma concentrations are sometimes obtained below 1 ng ml^{-1} in a 25-μl sample.

The majority of the drugs under investigation do not possess sufficient intrinsic fluorescence to allow an ultra-sensitive detection. Therefore, a chemical derivatisation reaction (covalent labellings procedure) should be applied. Derivatisation reactions can be performed before or after the LC separation both in on-line or off-line mode with the chromatography [5]. All derivatisation modes have their own advantages and disadvantages and consequently the proper mode will be strongly dependent on the problem in question.

The emphasis in this study is on the development of a selective indirect pre-chromatographic derivatisation procedure for the carboxylic acid function and subsequent LC separation, applying conventional (CIF) or laser induced fluorescence (LIF) detection of the resulting derivatives [6]. Furthermore, the possibilities of sample pretreatment, necessary for the bioanalysis of the analytes, using the analysis of morphine-3-β-D-glucuronic acid in rat plasma samples as an example, and the possibilities of constructing a laser based detection device are briefly discussed [7].

Experimental

Materials

Diethyl ether, ethyl acetate, dichloromethane, *N*,*N*′-dimethylformamide, methanol, and acetonitrile were obtained from Baker Chemicals (Deventer, The Netherlands). Tetrabutylammonium (TBA^+) bromide, tetramethylammonium (TMA^+) bromide, triethylamine, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED.2HCl), fluorescein, ethylenediamine, and 9-(hydroxymethyl)anthracene (HMA) came from Janssen Chimica (Beerse, Belgium). Morphine-3-β-D-glucuronide (MG), 3-β-oestradiol-17-β-D-glucuronide, α-naphthyl-β-D-glucuronide, mentholglucuronide, *p*-nitrophenyl-β-D-glucuronide, and monodansylcadaverine were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 2-Bromo-1-methylpyridinium iodide (BMP) was synthesized as previously described [8] and *N*-(1-fluorescein)ethylenediamine (FEDA) was synthesized, from fluorescein and ethylenediamine, applying the BMP procedure according to reference [6] and purified using the two-column cation-exchange/reversed-phase (CE/RP) system described in the same reference. Etoposideglucuronide, and fenoterolglucuronide were isolated from patients' urine or rat intestinal cells [6]. NED was extracted from 1 M sodium hydroxide, containing 1 mg ml^{-1} of NED.2HCl, by three successive extractions with equal volumes of dichloromethane.

The other reagents came from different sources and were used without further purification. Throughout the study deionized water (Milli Q Water Purification System, Millipore, Bedford, MA, U.S.A.) was applied.

Apparatus

The LC-LIF (Fig. 1) system consisted of a high pressure pump model 2150 (LKB Products, Bromma, Sweden) combined with a modified model 7125 Rheodyne

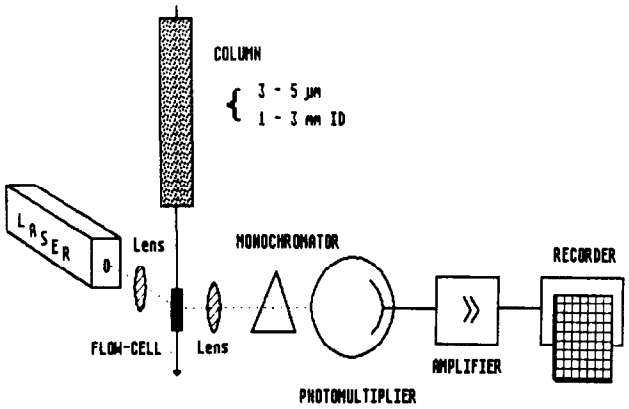

Figure 1
Liquid chromatographic — laser induced fluorescence detection system.

(Berkeley, CA, U.S.A.) fixed volume (20 µl) injector. In this modified injector the sample was introduced by means of a nitrogen flow (100 ml min^{-1}) instead of the normally applied syringe [9]. Before injection the samples were stored in capped polyethylene (1.0 ml) injection vials and transported through a stainless steel capillary (0.1 mm i.d.) from the vial to the injection valve.

The analytical column was either a glass cartridge (100 × 3.0 mm i.d.) packed with 5 µm ChromSep C_{18} (Chrompack, Middelburg, The Netherlands) particles or a stainless steel LiChrosorb C_{18} (30 cm × 3.9 mm i.d.) column packed with 10 µm particles (Merck, Darmstadt, FRG) in combination with a LiChrosorb Si-60 (30 cm × 3.9 mm i.d.) precolumn packed with 10 µm particles (Merck). The column switching was performed with a nitrogen-activated 7000 psi sample injection valve (Valco Instruments, Houston, U.S.A.) controlled by a digital valve interface (Valco) and a SP 4000 central processor (Spectra Physics, Santa Clara, CA) [6]. Chromatography was performed in all cases at 294 K and at a flow rate of 1.0 ml min^{-1}.

Detection of analytes was performed with a non-cooled model RCA 4517 photomultiplier tube (RCA, Harrison, NJ, U.S.A.) which was connected to a high voltage supply (1400 V) (model 244, Keithley Instruments, Cleveland, OH, U.S.A.) and a current amplifier (gain 10^5–10^7, rise time 10 ms) (model 427, Keithley Instruments). The obtained signals were recorded by a model BD-41 recorder (Kipp and Zonen, Delft, The Netherlands) equipped with a low-pass filter with an RC time of 1 s.

Excitation of samples was performed with a continuous-wave argon-ion gas laser (model 2015-03, Spectra Physics, Mountain View, CA, U.S.A.). The visible mode of the laser was applied at 488 nm, with an aperture of 12 and an output power of 2.0 W, and in the ultraviolet mode the 351.1–363.8 multiline was applied with an aperature of 12 and an output power of 100 mW.

The optical system consisted of a liquid light guide (1000 × 5.0 mm i.d.) with a numerical aperture of 0.47 and an acceptance angle of 56° (Oriel Corporation, Stratford, CT, U.S.A.). At the photomultiplier end of this optical fibre a plano convex fused silica lens (Oriel Corporation) (diameter 11 mm, focus length 19 mm) was fixed and at the flow-cell end of the fibre, two identical plano convex lenses were placed. The position of the lenses was manually optimised to obtain the highest signal. Between the fibre and the photomultiplier an interference filter with a transparency of 33% at 515 nm with a band-

width of 8 nm (Melles Griot, Irvine, CA, U.S.A.) or an interference filter with a transparency of 40% at 420 nm and a band-width of 10 nm (Melles Griot) was placed.

Between the laser and the optical system the flow-cell was placed, connected with the analytical column by means of a fused silica capillary (500 × 0.1 mm i.d.), allowing fluorescence measurements in the in-plane 90° geometry with a 25 μl model 176.70 QS cell (Hellma, Müllheim, F.R.G.), using an optical pathlength of 1.5 mm and an aperature of 11 × 1.5 mm.

CIF detection was accomplished with a Perkin–Elmer (Beaconsfield, U.K.) model 650 fluorescence detector (slit width 10 nm; 150 BC xenon power supply) for the analysis of the amine probes and the corresponding derivatives or an LS-4 (Perkin–Elmer) fluorescence detector (slit width 10 nm) for HMA and the HMA derivatives.

Methods

The sample pretreatment and the derivatisation procedure for the analysis of glucuronic acid conjugates are discussed in full detail in earlier studies [6, 9].

The slightly modified procedure for the analysis of MG in rat plasma samples was as follows: a 25 μl sample was mixed with 100 μl water and extracted with 1.0 ml of dichloromethane. The resulting water fraction was applied to a BondElut C_{18} cartridge (Analytichem International, Harbor City, CA, U.S.A.). After washing of the cartridge with hydrochloric acid (3 ml, 0.1 M), MG was eluted with 3 ml of methanol containing 5% (w/w) triethylamine. After evaporation to dryness, the residue was dissolved in 40 μl *N,N'*-dimethylformamide and derivatised according to the described procedure using NED as the fluorescence probe [6]. After evaporation to dryness of the derivatisation mixture the residue was dissolved in 50 μl of the eluent and 10 μl was injected onto CE silica system.

Discussion

Sample pretreatment procedure

For the analysis of glucuronides in plasma and urine samples, the discussed derivatisation technique requires a sample pretreatment procedure to isolate the analyte from the matrix and to transport it to an apolar (organic) solvent to allow quantitative derivatisation of the analyte.

Liquid–liquid extractions with relatively polar extraction solvents (e.g. diethyl ether, ethyl acetate), ion-pair extractions (e.g. TBA^+) or solid phase isolations with the XAD-2 material will result in low recoveries and unacceptable blank chromatograms.

The most suitable pretreatment for the majority of the glucuronides is the combination of a pre-extraction with dichloromethane and a RP solid phase isolation of the remaining aqueous fraction [6]. Subsequently, the glucuronide containing fraction is evaporated to dryness and after dissolution of the residue in a dichloromethane/*N,N'*-dimethylformamide mixture an aliquot is derivatised according to the procedure described below.

The described sample clean-up procedure can be applied for plasma and urine samples of humans as well as rats.

Covalent labelling procedure for glucuronides

The covalent labellings procedure for the carboxylic acid function of the glucuronide is based on the formation of an amide or an ester by means of a selective indirect pre-chromatographic derivatisation procedure.

After deprotonation, by an organic base, the carboxylic acid function is activated with BMP. The activated acid reacts in the following step with the fluorescence probe, a fluorescent amine (e.g. dansylcadaverine, NED, FEDA) or an alcohol (e.g. HMA) and the derivative, an amide or an ester is formed (Fig. 2).

The method is selective for the carboxylic acid function, because both the carbonyl and the hydroxyl functions of the acid are involved in the reaction. A number of simple mono-carboxylic acids [8] and glucuronic acid conjugates (Table 1) have been derivatised with this procedure and the derivatisation yield of all the tested analytes with the listed fluorescence probes (HMA, NED, FEDA, dansylcadaverine) was over 95%. Except for analytes possessing a carboxylic acid function, a number of solutes with other acidic functions (e.g. phenol, thiol, hydroxyl, imide) have been processed, but no derivatives could be detected [8].

Another feature of this procedure is that by varying the structure of the probe, except for the reactive amine or hydroxyl function, the derivatisation reaction can be used in combination with a number of detection principles (e.g. absorbance, amperometric, chemiluminescence, electron-capture, fluorescence, nitrogen-phosphoric).

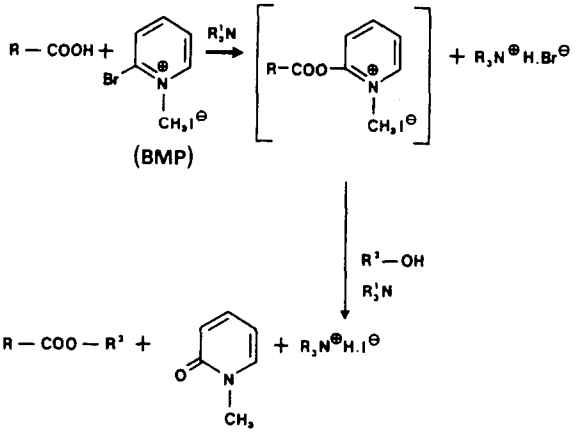

Figure 2
Reaction sequence of coupling of the carboxylic acid function with alcohols after activation with 2-bromo-1-methylpyridinium iodide.

Table 1
Capacity factors of glucuronide derivatives with *N*-(1-naphthyl)ethylenediamine as fluorescence probe. Eluent, 65% (w/w) methanol–35% (w/w) water with 2 mM TBA^+, 2 mM disodium citrate and a pH of 5.3 of the water phase

Compound	CE/RP	k' CE
Oestradiol-17-glucuronide	2.71	
Etoposide-glucuronide	1.17	
Fenoterol-glucuronide		2.01
Glucuronic acid	0.66	
Menthol-glucuronide	9.57	
Morphine-glucuronide		1.78
Naphthyl-glucuronide	2.11	
p-Nitrophenyl-glucuronide	1.99	

The derivatisation procedure itself is simple. After dissolution of the analyte, the activator (BMP), the base catalyst, and the fluorescence probe in dichloromethane, acetonitrile or *N,N'*-dimethylformamide, aliquots of these solutions are mixed and reacted for 5 or 30 min at ambient temperature (Fig. 2) [6, 8]. The reaction time depends on the choice of label. In case an amine is used as the fluorescence probe a reaction time of 5 min is sufficient, while for the alcohol probes reaction times of 30 min are needed. Subsequently the mixture is evaporated to dryness and after dissolution of the residue in the eluent, an aliquot is injected into a suitable LC system.

Chromatography of glucuronic acid conjugates

The modification of the injection device is needed to avoid adsorption of the analytes onto glass surfaces. This irreversible adsorption is especially troublesome with determinations on less than 1 pg of the analyte.

Whenever an alcohol is used as the fluorescence probe, the derivatives are analysed with a simple RP system [8] in combination with a mobile phase consisting of a mixture of methanol and water. When an amine is applied as the fluorescence probe the two-column CE/RP column switching system is used in combination with an eluent consisting of methanol, an acidic aqueous buffer (e.g. citrate), and competing-ions (e.g. TBA^+, TMA^+) [6].

Applying this system the excess of the fluorescent probe (the amine) is retained on a CE column, with non-modified silica as the stationary phase and a methanol/buffer solution as the mobile phase. The derivative however, is not retained on this column and is analysed with the on-line coupled RP column. For both columns, in the injection as well as in the eluting mode, the same eluent is applied.

For the analysis of amphoteric glucuronides (e.g. MG, fenoterol), after derivatisation with an amine probe, this CE/RP system cannot be applied, because the amide of the amphoteric glucuronic acid conjugate and NED will be retained on the CE silica column. Therefore, these derivatives are analysed using only a non-modified silica column in the CE mode.

Non-modified silica can be applied as a weak cation-exchanger because at pH values over two the silanol functions will be deprotonated, resulting in a negative charge on the silica surface.

Laser induced detection in liquid chromatography

Applying LIF detection in LC the laser (in this study the argon-ion laser) is used as the light source, meaning that in addition to a complete LC system, one or more lenses (focussing of excitation and emission beams), a flow-cell, a monochromator or an interference filter, a photon-counting unit, and recording facilities must be present.

In general, in bioanalysis the application of standard chromatographic LC systems (stationary phase: 3–5 μm particles; column, 1–3 mm i.d.) is advantageous, hence conventional square quartz flow-cells (probe volume 2.6 μm) can be applied. Furthermore, it is not necessary to use expensive photon-counting or optical systems. A non-cooled blue/green sensitive photomultiplier in combination with an interference is sufficient (Fig. 1). The focussing lenses in the system can be replaced by a liquid light guide which allows more flexibility in the geometry of the optical train. The application of a monochromator is only advantageous if a number of different fluorescence probes, with strongly deviating excitation wavelengths, are used.

Fluorescence detection: conventional versus laser induced detection

The glucuronic acid derivatives are separated with a RP or CE/RP chromatographic system and are detected with CIF or LIF detection. The composition of the eluent is thus very important in order to obtain the required sensitivity.

For instance, for the NED as well as for the FEDA derivatives the pH of the mobile phase must be selected between 4 and 10. However, because silica based stationary phases are used, the latter pH may not exceed the value of 8. For a high detectability of the NED derivatives relatively high concentrations of the organic modifier are advantageous, while to increase the detection sensitivity of the FEDA derivatives low modifier concentrations are advisable.

For the other fluorescence probes different values are valid, of course, but these data should be established carefully for each individual probe.

The mentioned fluorescence probes can be used for CIF detection as well as LIF detection applying the argon-ion laser. For instance, FEDA with an excitation wavelength of about 490 nm, can be used in combination with the 488 nm emission line of the laser and the alcohol HMA (excitation 365 nm), and the amines NED and dansylcadaverine (excitation 335 and 355 nm respectively) can be used in combination with the 350–360 multiline of the laser. Furthermore, the probes are chosen in such a way that the intrinsic fluorescence sensitivity of the non-derivatised and derivatised probes are comparable [5].

Applying CIF detection the minimum detectable amounts, in biological samples, of the NED, the HMA, and the fluorescein (e.g. FEDA) derivatives are between 5 and 50 pg (Fig. 3). Applying LIF detection, using the ultraviolet mode of the argon-ion laser, these detection limits are about 200 fg for the HMA derivatives, which is a gain of over

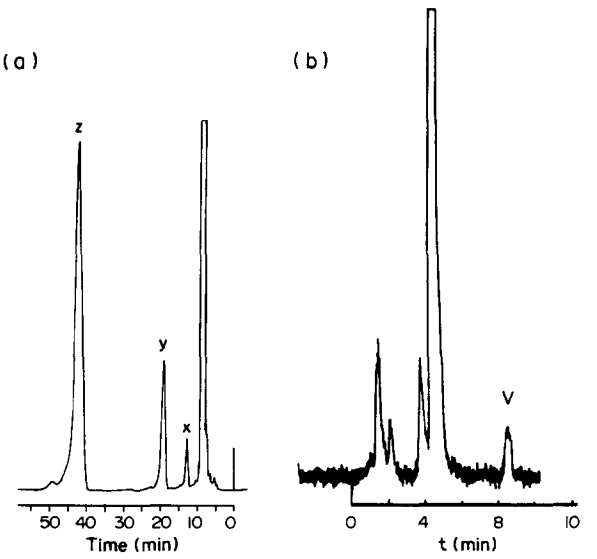

Figure 3

Chromatograms obtained after derivatisation of naphthylglucuronide (x), oestradiol-17-glucuronide (y), mentholglucuronide (z) (a), and morphineglucuronide (v) in a 25 μl plasma sample (b) with *N*-(1-naphthyl)ethylenediamine using conventional induced fluorescence detection. x, y and z represent the injection of about 200 ng, and v about 5 pg of the corresponding derivatives. x, y and z were separated with the CE/RP system and v with the CE system applying the eluent as described in Table 1.

two orders of magnitude. This relatively limited gain in sensitivity is due to the fact that the output power of the argon-ion laser in this wavelength area is limited to about 200 mW and to the occurrence of a relatively high background signal (matrix and system noise). Applying the visible mode of the laser both contributions to the background will be strongly decreased and in combination of output powers of about 2 W this will result in minimum detectable amounts of about 1 fg for the fluorophore fluorescein, which is a gain of four orders of magnitude in comparison with CIF detection (Fig. 4).

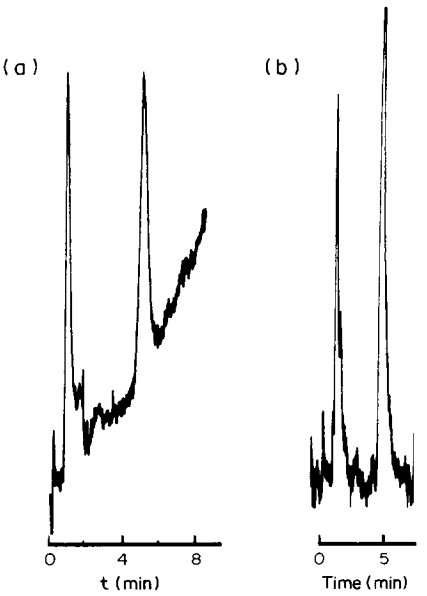

Figure 4
Chromatograms obtained after injection of 5 pg of hydroxymethylanthracene (a) and 25 fg of the fluorescein fluorophore (b) applying laser induced fluorescence detection. Hydroxymethylanthracene was separated with the RP system and an eluent consisting of methanol 65% (w/w) and water 35% (w/w). The fluorescein fluorophore was quantitated with a mobile phase of 65% (w/w) methanol and 35% (w/w) 0.01 M phosphate buffer (pH 5.5).

Analysis of morphine-3-β-D-glucuronide in rat plasma

MG, as an example of an amphoteric glucuronide, was analysed in 25 μl rat plasma samples (Fig. 3). The derivative was separated with CE silica system using a mobile phase consisting of 65% (w/w) methanol and 35% (w/w) water containing 2 mM TBA^+, 2 mM disodium citrate and a pH of 5.3 of the aqueous phase. The amide of MG and NED (k' 1.78) was well separated from the excess NED (k′ 0.41) and the derivatisation yield, after labelling of 500 ng of MG, was the same as described for other procedures. A calibration curve, after the described sample clean-up, showed good linearity; e.g. $y = -6.14\ (\pm 3.17) + 0.095\ (\pm 0.003)\ x$ ($r = 0.994$) was the equation for the calibration line after the analysis of nine samples containing 0.2–2.0 ng MG.

Conclusions

It can be stated that LIF detection using the visible mode of the argon-ion laser and a simple home-made apparatus, offers the possibility of ultra-sensitive detection of glucuronic acid derivatives, after derivatisation with one of the fluorescein derivatives.

Taking advantage of the application of standard chromatographic systems, a relatively inexpensive LIF detection apparatus can be constructed consisting of: a standard LC unit, including a conventional flow-cell, a condenser lens or an optical fibre, an

interference filter, a photomultiplier and an amplifier. The described home-made system allows detection limits of about 1 fg after 20-μl injections of the fluorophore.

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[*Received for review 24 September 1987; revised manuscript received 9 October 1987*]