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Quinoxalinone derivatization of biological carboxylic acids for detection by peroxyoxalate chemiluminescence with high-performance liquid chromatography

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Abstract

A quinoxalinone fluorescent tag is evaluated as a carboxylic acid derivatizing reagent for detection by peroxyoxalate chemiluminescence. The synthetic procedure for the quinoxalinone was modified to give a yield that is significantly increased over that reported previously. The new conditions use less hazardous reagents, and produce a final product greater than 97% pure, without the need for intermediate clean-up steps. The derivatization reaction is also modified to give increased yields of greater than 85% compared to 74% obtained previously. The post-column chemiluminescence reaction conditions are optimized to give detection limits of 500 attomole/injection–10 times lower than the fluorescence previously obtained. The reagent is used for the first time on a plasma sample extract. Typical method precision is 4%.

1. Introduction

Several criteria are important in the development of a precolumn derivatization reagent: commercial availability; moderate derivatization conditions; no interferences in derivatization or detection; stability; and suitability for the detection of low analyte levels. Several types of molecules have been investigated as tagging reagents which meet some, but not all of these criteria. These include coumarins [1,2], a series of benzoxadiazoles [3], 1-bromoacetylpyrene [4], anthryldiazomethane [5] and 2-bromo-6-methoxynaphthalene [6]. Other fluorophors have also been used to improve selectivity and sensitivity including 4 - (bromomethyl) - 7 - methoxycoumarin [7,8] and 7 - (diethylamino) - 3 - [4 - ((iodoacetyl)amino)phenyl] - 4 - methylcoumarin [9]. Several groups have investigated the use of quinoxalinone fluorophors as tagging reagents for carboxylic acids [10–12]. In contrast to other reagents, quinoxalinone tagging is done at ambient temperature, reaction times are shorter and the reagent is more selective [13]. The most recent work done by Yamaguchi *et al.* showed 6,7 - dimethoxy - 1 - methyl - 2(1*H*) - quinoxalinone - 3 - proprionylcarboxylic acid hydrazide (quinoxalinone) to be a particularly sensitive tagging reagent and reported detection limits of 3 fmol/injection or 300 fmol/ml [14]. Although not commercially available, it meets all of the other criteria stated above. In addition, it is a very selective reagent for carboxylic acids.

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Yamaguchi *et al.* reported that quinoxalinone does not react with α-keto acids, 17 α-amino acids, alcohols, sugars, amines, aldehydes, ketones, phenols and sulphydryl moieties. This makes it not only a very sensitive, but also a very specific tagging compound, which minimized sample cleanup.

Chemiluminescence detection of fluorescent compounds was first reported for HPLC by Kobayashi and Imai [15]. The most sensitive fluorophors for peroxyoxalate chemiluminescence are aminopolynuclear aromatics [16–18] and coumarins [19]. Their sensitivity has been attributed to a combination of redox and fluorescent properties. Peroxyoxalate chemiluminescence is a base catalyzed reaction. Therefore the given fluorophor must be insensitive to, or reach a maximum emission around pH 7. The quinoxalinone chosen exhibited all of these properties required for a potentially sensitive peroxyoxalate chemiluminescence reagent.

Here we report the modification of the quinoxalinone synthesis, evaluation of the derivatization conditions for quinoxalinone tagged carboxylic acids, optimization of the peroxyoxalate chemiluminescence post-column reaction conditions and matrix effects in a clinical sample extract.

2. Experimental

2.1. Apparatus

Proton nuclear magnetic resonance spectra were obtained on a General Electric QE-300 spectrometer (Fremont, CA, USA) at 300 MHz. Hydrogenation was done on a Parr Model 3911 hydrogenator (Parr, Moline, IL, USA).

The peroxyoxalate chemiluminescence HPLC system shown in Fig. 1 consisted of two M-6000A high pressure mixing pumps (Waters, Milford, MA, USA), a Model 680 gradient controller (Waters), a 10-μl injection valve (Rheodyne, Cotati, CA, USA), a Zorbax ODS 250 × 4.6 mm I.D., 5 μm packing (MAC-MOD, Chadds Ford, PA, USA) and a Model 980 fluorescence detector equipped with a 25-μl flow

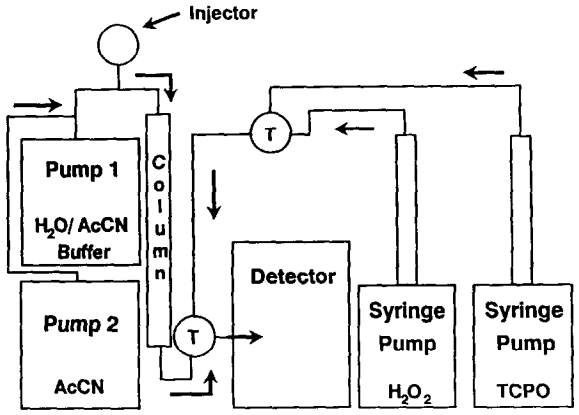

Fig. 1. Schematic of high-performance liquid chromatograph with peroxyoxalate chemiluminescence post-column detection (see Experimental section for description of components).

cell (ABI, Foster City, CA, USA). The PMT voltage was −1500 V. The post column reagents were added using two Model LC-2600 syringe pumps (Isco, Lincoln, NE, USA). These reagents were mixed initially in a mixing tee (VICI Valco Instruments, Houston, TX, USA) followed by a 6.9-bar backpressure regulator (Upchurch Scientific, Oak Harbor, WA, USA) then added to the column effluent in a second mixing tee (Valco). A 30 cm × 0.25 mm I.D. piece of stainless steel tubing was used as a reaction delay coil. Data was collected and integrated using a Model SP4290 integrator (Spectra-Physics, San Jose, CA, USA).

2.2. HPLC conditions

The ODS column described above was used for all work in this paper. The mobile phase was run in a gradient mode. The two solvents designated A and B were acetonitrile–water (40:60, v/v) with 25 m*M* imidazole (A), and acetonitrile (B). The gradient used for the fatty acids and arachidonic acid metabolites at 1.2 ml/min was a 50A:50B 10-min linear gradient to B, hold 5 min, at a flow-rate of 1.2 ml/min. The gradient for the valproic acid work was the same as that above, but started at 75A:25B. The bis(2,4,6-trichlorophenyloxalate), TCPO, used was 25 m*M* at a flow-rate of 1.0 ml/min and the 0.5 *M* peroxide had a flow-rate of 0.5 ml/min. The

solvent used for the peroxide buffer was water–acetonitrile (10:90, v/v) and for the TCPO was ethyl acetate.

2.3. Chemicals

All chemicals were reagent grade unless otherwise specified.

Synthesis of quinoxalinone: 1,2-Dimethoxy-4,5-dinitrobenzene (Lancaster, Windham, NH, USA), 83% platinum (IV) oxide, 2-ketoglutaric acid, 1.0 *M* hydrogen chloride in anhydrous diethyl ether and methyl iodide (Aldrich, Milwaukee, WI, USA) were used. Zero grade hydrogen (AGL, Clifton, NJ, USA) was used in the reduction step. All solvents, dimethylformamide (DMF), dimethylsulfoxide (DMSO), anhydrous ethanol and ethyl acetate were HPLC grade and hydrazine monohydrate was reagent grade (Fisher Scientific, Springfield, NJ, USA).

Tagging reaction: Coupling reagents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1,1′-carbonyldiimidazole and N,N′-dicyclohexylcarbodiimide (DCC) were used as potential coupling reagents (Sigma, St. Louis, MO, USA). Acetonitrile and methylene chloride were HPLC grade (Fisher). The other solvents used were mentioned above.

Peroxyoxalate chemiluminescence HPLC: Acetonitrile, ethyl acetate and methanol were HPLC grade and 30% hydrogen peroxide was reagent grade (Fisher). Bis(2,4,6-trichlorophenyloxalate) was received from A. Mohan (American Cyanamid, Pearl River, NY, USA) and used without further purification. Imidazole was used for the buffer (Aldrich).

Fatty acids standards, caproic (C_8), pelargonic (C_9), capric (C_{10}), hendecanoic (C_{11}) and lauric (C_{12}) were obtained from Aldrich and the hydroxyeicosanoic acids were obtained as solutions in ethanol (Cayman Chemical, Ann Arbor, MI, USA). Bovine plasma and valproic acid were purchased from Sigma.

The synthetic procedure used for quinoxalinone is given in Fig. 2. It was based on work done by Yamaguchi *et al.* [14]. The individual steps are given below. Note: abbreviations used for the NMR data are s = singlet, t = triplet.

Compound I was prepared by pre-reducing 100 mg of PtO_2 in ethyl acetate–ethanol (3:2, v/v) for 1 h at 2 bar with zero grade hydrogen. Then 1.0 g (4.4 mmol) of 1,2-dimethoxy-4,5-dinitrobenzene in 15 ml of ethyl acetate was added. The mixture was reduced overnight (16 h) using hydrogen at 2 bar. An additional 50-ml volume of ethyl acetate was added and the

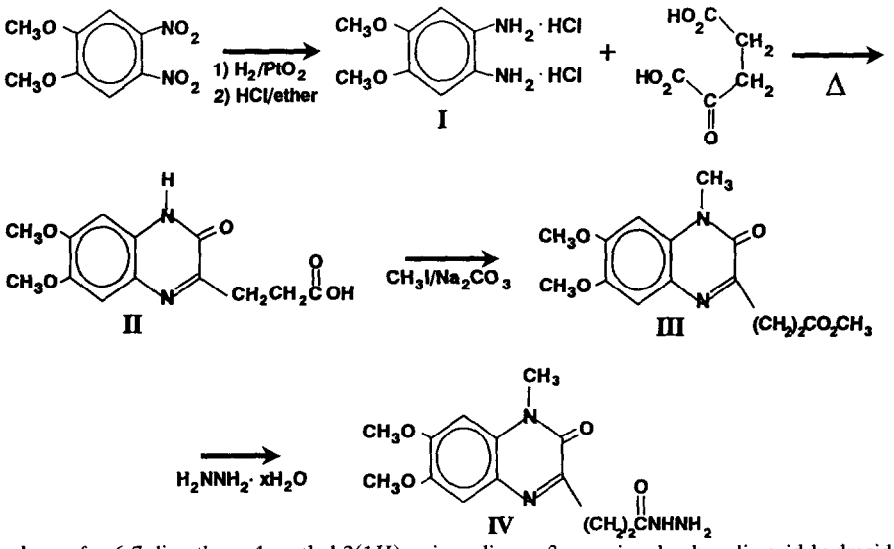

Fig. 2. Synthesis scheme for 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-proprionylcarboxylic acid hydrazide (conditions for synthesis are given in the Experimental section).

resulting solution was filtered through Celite. A 20-ml volume of anhydrous 1.0 *M* hydrogen chloride in diethyl ether was added to the filtrate. The resulting precipitated hydrochloride salt was filtered and dried overnight in a vacuum oven at 40°C. The purity of the product was determined to be 97% by HPLC with 2% of the dinitrobenzene starting material and 1% other impurities. The melting point was 250–251°C and the NMR (in D_2O) was δ (ppm) 3.79 (s, 6H), 6.83 (s, 2H). As this data was consistent with the desired material, and HPLC and melting point indicated the purity was high, no additional purification was done. The yield was 78%. This work was based on that done by Dave *et al.* [11] and differs from their procedure by (a) pre-reduction of the PtO_2, and (b) use of commercially available hydrogen chloride in ether.

Compound II was prepared by reacting the amine hydrochloride with 2-ketoglutaric acid as done by Hara *et al.* [13]. The yield was 83%. In order to confirm that we had the desired fluorophor, a fluorescence spectrum was run. A corrected spectrum on compound II gave excitation and emission maxima of 368 and 445 nm, respectively. This is in agreement with the 360 and 440 nm reported.

To prepare compound III 500 mg of compound II were transferred to a foil covered flask to which 600 mg of sodium carbonate, 1.25 ml of methyliodide, and 25 ml of DMF–DMSO (10:90, v/v) was added. The reaction mixture was stirred for 3 h. The resulting product was precipitated from 125 ml of 10% brine, washed with additional brine and dried overnight in a vacuum oven at 60°C. The melting point was 176–178°C and the product was 92% pure as determined by area measurement using HPLC. The NMR (in d_6-DMSO) showed δ (ppm) 2.77 (t, 2H), 3.02 (t, 2H), 3.53 (s, 3H), 3.62 (s, 3H), 3.81 (s, 3H), 3.90 (s, 3H), 6.96 (s, 1H), 7.18 (s, 1H). Elemental analysis calculated for $C_{15}H_{18}N_2O_5$: C: 58.82%, H: 5.92%, N: 9.15%; found C: 59.09%, H: 5.89%, N: 9.00%. The yield was 77%.

The synthesis of this material was reported to be done in diazomethane followed by a silica gel column cleanup which gave a 62% yield [14]. At the levels indicated in this work (1500 mg in 100 ml anhydrous methanol), and down to 1 mg/ml we observed that the compound precipitated. Although the reaction could be done under these conditions, an alternative methylation approach was sought which would be less reactive and less hazardous. The reagent chosen was methyliodide. Based upon work done by Ireland *et al.* [20], a polar solvent system was used. DMF as a co-solvent in DMSO improved reaction yields dramatically.

To prepare compound IV 1.0 ml of 45% hydrazine monohydrate was added to 0.50 g of compound III. The mixture was heated in a glycerol–water bath (5:95, v/v) at 100°C until the first drop fell from the bottom of the condenser. Then 5.0 ml of absolute ethanol was added and the reaction mixture refluxed for 2 h. The final product was precipitated from the mother liquor in an ice-water bath. The resulting precipitate was filtered into a flask containing 10 ml of acetone to quench the excess hydrazine. The product was dried in a vacuum oven overnight at 60°C. The purity as determined by HPLC was 94% by and the melting point was 204–205.5°C. The NMR (in d_6-DMSO) was δ (ppm) 2.45 (t, 2H), 2.47 (t, 2H), 3.60 (s, 3H), 3.82 (s, 1H), 3.94 (s, 3H), 4.13 (broad s, 2H), 6.94 (s, 1H), 7.18 (s, 1H), 8.96 (broad s, 1H). The triplet at 2.47 was confirmed (due to an interference from the DMSO) by running a dilute solution in $CDCL_3$. Elemental analysis calculated for $C_{14}H_{18}N_4O_4$: C: 54.90%, H: 5.92%, N: 18.29%; found: C: 54.86%, H: 6.12, N: 18.30. The yield was 79%. This final material was synthesized using a modified procedure from Shriner *et al.* [21]. Compared to the original work 50 times less hydrazine was used and comparable yields were obtained.

2.4. Optimized derivatization conditions

A 100-μl volume of the acid (the HETEs, which are sold in ethanol, were evaporated under nitrogen and reconstituted in methylene chloride), 100 μl of a 50 mg/ml solution of EDC, 20 μl of 20 mg/ml pyridine and 1 mg/ml quinoxalinone were placed in a 2.0-ml vial. The

mixture was allowed to react for 30 min at ambient temperature. The solution was injected directly onto the HPLC.

2.5. Plasma extraction conditions

Into a 10-ml vial, a 200-μl sample of plasma was spiked with 100 μl of valproic acid/nonanoic acid stock solutions prepared in 0.5 *M* NH_4OH. In order to denature the plasma proteins, 50 μl of 10% $HClO_4$ in water was added. Then 2.0 ml of *n*-butylchloride–methylene chloride (75:25, v/v) extraction solvent was added (used because a sharp boundary is obtained between this solvent and the aqueous plasma layer). The sample was vortex-mixed for 30 sec. A 1.0-ml aliquot was removed and placed into a 1.4-ml microcentrifuge tube. The extract was vortex-mixed for 3 min at 16 000 *g*. Then 100 μl of the organic layer was derivatized using the procedure outlined above, but 1.0 ml of acetonitrile was used rather than 300 μl.

3. Results and discussion

3.1. Synthesis modifications

Using the modifications reported in the Experimental section, 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-proprionylcarboxylic acid hydrazide was synthesized with an overall reaction yield of 39%. This was a 40% increase over the 28% previously obtained. The purity was 94% by HPLC and therefore no additional cleanup was done.

3.2. Derivatization of carboxylic acids

Various types of carboxylic acids were used in evaluating the derivatization conditions, chemiluminescence reaction optimization and matrix effects. Several arachidonic acid metabolites (5-HETE, 12-HETE and 15-HETE) were used in the evaluation of the derivatization conditions since these molecules may show side reactions. Because they are easy available fatty acids were then used to optimize the chemiluminescence reaction. Finally, matrix effects were investigated using valproic acid, an anti-hypertensive, in clinical concentration ranges.

Effects of reaction conditions

Preliminary attempts at tagging stearic acid using an aqueous based solvent system were not successful. This was attributed to the hydrolysis of the coupling agent under aqueous conditions. Therefore non-aqueous based systems were investigated.

EDC in methylene chloride with pyridine was determined to be the only coupling reagent/solvent/catalyst combination of those tried (including DCC and CDI in 5 different solvents and two other catalysts), which gave the desired fluorescent tagged carboxylic acid. This reaction was confirmed for the remaining eicosanoids and a series of straight chain fatty acids. The 5-HETE, a 5-hydroxyacid, lactonized during the reaction under anhydrous conditions. This was later confirmed by retention time comparison of the 5-HETE lactone standard purchased from Cayman.

Optimization of derivatization conditions

The conditions for derivatization given in the Experimental section were optimized by means of fluorescence intensity using HPLC. A central composite design optimization was used for three variables: concentrations of pyridine, EDC and fluorophor. These final conditions utilized fifteen times less fluorphor and eight times less EDC than previously reported. This difference was attributed to the use of non-aqueous solvents which reduces hydrolysis and therefore the need for more concentrated reagents.

Derivatization yield can be calculated by comparing the response of the derivative to that of the free fluorophor. It is reasonable to assume that the analyte would have little effect on the fluorescence efficiency. This comparison gave yields of 85–90%. This improvement of 10–15% over that reported earlier, was also attributed to the lack of hydrolysis.

Central composite design optimization of the peroxyoxalate chemiluminescent reagents

There are several key parameters involved in the optimization of peroxyoxalate chemiluminescence detection. These include TCPO concentration, H_2O_2 concentration, aqueous content of the mobile phase and post-column flow-rates. The ester and peroxide flow-rates and concentrations were optimized using a central composite design. Provided that the pH used is close to the optimum, it will have a minimal effect on the signal. The most important parameter in controlling peroxyoxalate chemiluminescence kinetics is peroxide concentration. Therefore the pH used, 7.0, was found to be in the midrange of the optimum pH (6.5–7.5) when TCPO is used as the ester [22,23]. As the water content of the mobile phase affects baseline drift and causes signal loss and precipitation of the TCPO, the tolerable water level was investigated. This maximum was found to be 30% in the mobile phase before precipitation occurred. The concentrations and relative flow-rates of the peroxide and ester were similar to those reported [22].

Precision, detector linearity and detection limits

The linearity of the detection was determined for 12-HETE and 15-HETE over the range of 20 pg/ml to 80 ng/ml and was confirmed at the 95% confidence level using an F-test. The precision of the derivatization was ± 4.2% relative for 3 injections at the 7 ng/ml level with an injection precision of 2.3%. The detection limit at 3 S/N (peak-to-peak noise) was 4 pg/ml which was at least ten times lower than that reported by radioimmunoassay [24], mass spectrometry [25], or fluorescence detection [14].

3.3. Plasma matrix effects

Influence of plasma matrix on derivatization

The utility of this quinoxalinone as a fluorophor tag for clinical samples has not been demonstrated. We investigated the extraction and derivatization of valproic acid, an anticonvulsant.

Effects of the aqueous content of the mobile phase on separation efficiency and the post-column reaction

All post-column reaction conditions used (peroxide and TCPO concentrations, flow-rates, etc.) were those given above in the experimental section. The PMT voltage was −1500 V for this work. Although the same column was used as above, the separation of the tagged valproic acid from the excess fluorophor required the use of a higher aqueous/organic ratio in the mobile phase. A solid-phase extraction cleanup procedure was tried to reduce the excess fluorophor, but due to the similar polarity of the excess tag and tagged valproic acid, separation was not initially attained. In order to obtain the desired separation the mobile phase had to have a 60% aqueous content. At this level, however, precipitation of the TCPO was occurring. By inserting a switching valve between the column and injector, the column effluent was diverted until 5.5 min after the start of the run when the aqueous content had decreased to 25% and the fluorophor had eluted. This technique not only alleviated the precipitation problem, but removed the fluorophor from the detection system eliminating the need for any pre-column clean up.

Matrix assay of valproic acid by peroxyoxalate chemiluminescence detection

Nonanoic acid (Aldrich), which has already been used as an internal standard in valproic assay from plasma [26], was chosen as the internal standard. The partition coefficients for the two acids were similar as equal peak areas were obtained for equivalent concentrations of nonanoic acid and valproic acid upon extraction from plasma. Nonanoic acid was also chosen as it was baseline resolved from the co-extracted impurities observed at low levels.

Using the plasma extraction conditions mentioned in the Experimental section, extracts of blank and spiked plasma samples were prepared and derivatized. The corresponding chromatograms are given in Fig. 3a and b, respectively. The column effluent was switched into the post-column at 5.5 min. At this point a slight negative drift was observed in the baseline.

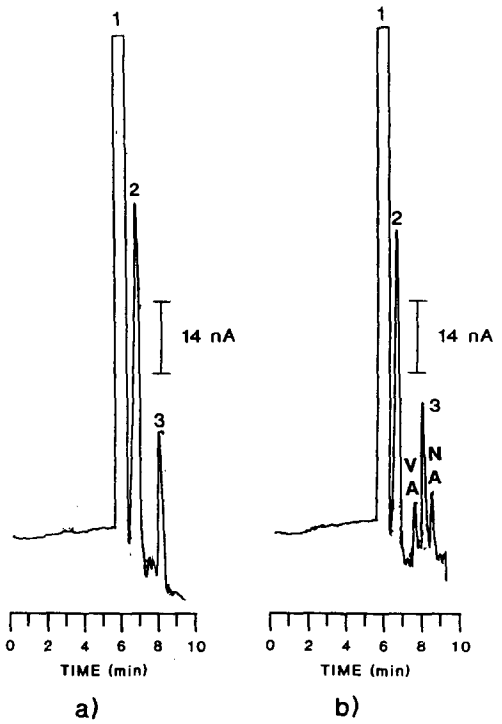

Fig. 3. Chromatograms showing derivatized valproic acid samples: (a) derivatized plasma blank, (b) extracted spiked plasma (1.0 ng tagged valproic acid or 5.2 μg/ml in plasma with 5.0 μg/ml nonanoic acid). Peaks 1 and 2 are from the excess quinoxalinone, peak 3 is a concomitant from the plasma extract, peaks labeled NA and VA are the nonanoic and valproic acid, respectively (see experimental section for HPLC conditions).

This drift can be attributed to the dilution which occurs upon switching the column flow and the resultant addition of the column effluent to the post-column reaction.

Method recovery, precision and linearity

The clinical range for valproic acid as an anticonvulsant drug is 40–150 μg/ml [27]. Method recovery and precision was tested at 62 μg/ml in plasma. Recovery was greater than 95%. Precision for 5 replicate extractions/derivatizations gave a relative standard deviation of 4.0% at the 95% confidence level. Linearity was confirmed at the 95% confidence level using an F-test over a range of 513 μg/ml (2.0 ml was used for the final volume at this level) to 1.03 μg/ml in plasma. Note: this corresponds to a final concentration in acetonitrile of 5.2 μg/ml to 10.4 ng/ml. This derivatization was done at levels 50 times less than could be done with coumarin derivatization as no interferences were detected at the 1 μg/ml range [9]. Interferences were not found to pose a problem until the 50–100 ng/ml range. As was demonstrated in our preliminary work, a solid-phase extraction should reduce the interferant further.

4. Conclusions

The compound 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-proprionylcarboxylic acid hydrazide has been evaluated for use as a fluorophor tag in peroxyoxalate chemiluminescence detection of carboxylic acids. Detection limits of 4 pg/ml for the HETEs (500 attomole per injection of the derivative), ten times lower than those obtained by Yamaguchi *et al.* were observed. The quinoxalinone synthesis was modified to increase the overall yield of the 4-step synthesis from 28% to 39%, and the derivatization reaction yield has been improved from 74% to 90%. The derivatization procedure uses significantly less reagent (8 times less EDC and 15 times less fluorophor) than previously. The derivatization has been used on a bovine plasma extract and found to give reproducible recoveries to 100 times below clinical concentrations with a relative precision of 4.0% using an external standard and <2% using an internal standard. Sample clean-up and the chromatographic separation were improved by using a switching valve technique.

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